The Maladaptive Response of Adipose Tissue to High Fat Diet Feeding

Approved by Supervisory Committee

_____
Philipp E Scherer, Ph.D.

_____
Rana Gupta, Ph.D.

_____
Rolf Brekken, Ph.D.

_____
Ralf Kittler, Ph.D.

_____
James Kim, M.D. Ph.D.

Dedication:

To My parents who started this all

To My grandparents who put me on this path

And to Anna and Lola for getting me through it

The Maladaptive Response of Adipose Tissue to High Fat Diet Feeding

By

Thomas Morley

Dissertation

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center

Dallas, Tx

May, 2016

Table of Contents

Acknowledgements

First and foremost I would like to thank my graduate school mentor Philipp Scherer. His unconditional and unbridled support throughout the entirety of my graduate school experience has changed who I am and how I conduct my life for the better. His infectious enthusiasm has allowed me to accomplish things I had only previously dreamed of. He is truly the center of all of this and I can only begin to try and thank him enough. Philipp you are my favorite. Thank you.

To Will Holland and Rana Gupta, from no obligation to anyone, you gave me guidance and support time and time again. Without your constant help, I would never have accomplished what I have. Your help means more than you will ever know and I promise to pay this forward

To my lab mates Jonathan Xia, Ingrid Asterholm, Joseph Rutkowski, Joshua Johnson as well as Lavanya Vishvanath, your help in these endeavors was essential at many critical junctures and I appreciate every moment of your help. Thank you.

And finally to my committee, thank you kindly for putting up with my less than perfect personal presentation style. You are gentlemen and scholars and I greatly appreciate the knowledge you have bestowed upon me.

Prior Publications

Vishvanath L, MacPherson KA, Hepler C, Wang QA, Shao M, Spurgin SB, Wang MY, Kusminski CM, **Morley TS**, Gupta RK. PdgfrB(+) Mural Preadipocytes Contribute to Adipocyte Hyperplasia Induced by High-Fat-Diet Feeding and Prolonged Cold Exposure in Adult Mice
Cell Metab. 2016 Feb 9;23(2):350-9. PMID: 26626462

Morley TS. Xia JY, Scherer PE. Selective enhancement of insulin sensitivity in the mature adipocyte is sufficient for systemic metabolic improvements
Nat Comm. 2015 Aug 5;6:7906. PMID: 26243466

Ye R, Holland Wl, Gordillo R, Wang M, Wang QA, Shao M, **Morley TS**, Gupta RK, Stahl A, Scherer PE. Adiponectin is essential for lipid homeostasis and survival under insulin deficiency and promotes B-cell regeneration
Elife. 2014 Oct 23;3. PMID: 25339419

Park J, **Morley TS,** Kim M, Clegg DJ, Scherer PE. Obesity and Cancer—mechanisms underlying tumour progression and recurrence
Nat Rev Endocrinol. 2014 Aug;10(8):455-65. PMID: 24935119

Wernstedt Asterholm I, Tao C, **Morley TS**, Wang QA, Delgado-Lopez F, Wang ZV, Scherer PE. Adipocyte Inflammation is essential for healthy adipose tissue expansion and remodeling
Cell Metab. 2014 Jul 1;20(1):103-18. PMID: 24930973

Xia JY*, **Morley TS*,** Scherer PE. The adipokine/ceramide axis: key aspects of insulin sensitization *equal contributions
Biochimie. 2014 Jan;96:130-9. Review. PMID: 23969158

Park J, **Morley TS**, Scherer PE. Inhibition of endotrophin, a cleavage product of Collagen VI, confers cisplatin sensitivity to tumors
EMBO Mol Med. 2013 Jun;5(6):935-48. doi: 10.1002/emmm.201202006. PMID: 23629957

Bachran C, **Morley T,** Abdelazim S, Fattah RJ, Liu S, Leppla SH. 2013 Anthrax toxin-mediated Delivery of the Pseudomonas exotoxin A Enzymatic Domain to the Cytosol of Tumor Cells via cleavable ubiquitin fusions
Mbio. Apr Apr 30;4(3):e00201-13. doi: 10.1128/mBio.00201-13. PMID: 23631917

Schafer JM, Peters DE, **Morley T**, Liu S, Molinolo AA, Leppla SH, Bugge TH. Efficient Targeting of head and neck squamous cell carcinoma by systematic administration of a dual uPa and MMP- activated engineered anthrax toxin
PLoS One. 2011;6(5):e20532. doi: 10.1371/journal.pone.0020532 PMID: 21655226

Liu S, Crown D, Miller-Randolph S, Moayeri M, Wang H, Hu H, **Morley T**, Leppla SH. Capillary morphogenesis protein-2 is the major receptor mediating lethality of anthrax toxin in vivo
Proc Natl Acad Sci U S A. 2009 Jul 28;106(30):12424-9. PMID: 19617532

List of Figures

Chapter 2 **Page**

Chapter 4

List of Abbreviations

AGPAT Acylglycerol-3-phosphate acyltransferase

DGAT- Diglyceride acyltransferase

FACS- Fluorescence activated cell sorting

Glut1/Glut4 Glucose transporter- 1 and 4 respectively

HFD- High Fat Diet

IHC- Immunohistochemistry

ITT- Insulin Tolerance Test

MCP-1- Monocyte Chemoattractant protein 1

MGAT –Monoglyceride acyltransferase

mRNA- Messenger RiboNucleic Acid

NEFA- Non-esterified fatty acids

Ogtt- Oral glucose tolerance test

PGC1-alpha- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPar-gamma- Peroxisome Proliferator activated receptor gamma

PRDM16- PR domain containing 16

TNF-alpha- Tumor Necrosis Factor alpha

UCP-1- Uncoupling protein 1

VEGF-A- Vascular Endothelial Growth Factor A

3t3L1- immortalized cell line capable of differentiating into adipocytes

Chapter 1

White Adipose Tissue

The importance of adipose tissue

The ability of organisms, from yeast to humans, to safely store energy as dense hydrophobic carbon chains secured away in lipid droplets represents an incredible evolutionary advantage. The further use of adipocytes as a professional lipid storage cells emphasizes the importance and benefit of safe long-term energy storage. As lipids are capable of acting intracellularly as both signaling molecules and detergents, there proper storage and sequestration is of pivotal importance if they are to be used for the maintenance of energy homeostasis. With the safe storage of energy dense lipids and their later partitioning to other metabolically active tissues, the production of ATP can continue to occur in multiple organs during periods of food deprivation. With this in mind, many early investigators saw the importance of adipose tissue, though its true importance was not recognized till relatively recently.

Classical adipose tissue function - A storage vessel

Adipose tissue stores lipids for times of nutrient deficiency. In the fed state, glucose coming in to the blood from the gastrointestinal tract triggers the release of insulin. Insulin is capable of acting on the adipocyte via its receptor activating a signal transduction cascade which results in activation of AKT. This activation of AKT leads to downstream activation of Rab and RAC1 which allows the glucose transporter GLUT4 to be inserted into the plasma membrane (1). With Glut4 inserted into the membrane, glucose is able to flow into adipocytes, resulting in an increase in intracellular glucose concentrations. Insulin further acts to enhance the rates of glycolysis within the adipocyte driving the production of acetyl-CoA (2). Acetyl-CoA carboxylase (primarily ACC1 in the adipose tissue) then drives the production of malonyl-CoA from acetyl-CoA. Malonyl CoA can then be used as a building block by fatty acid synthase (FAS) to produce long chain fatty acids such as palmitic acid (3). Palmitic acids are then conjugated onto

a glycerol backbone via the actions of multiple enzymes including GPTA, AGPAT, lipin, MGAT and DGAT resulting in the formation of triglycerides (4).

While *de novo* lipogenesis is a method for storing excess carbohydrates consumed in the diet, adipocytes can also take up fatty acids directly from the blood as they circulate in the form of chylomicrons and VLDL. Via the action of lipoprotein lipase, a lipase secreted from parenchymal cells such as muscle and adipose tissue, but attached to the surface of capillaries via GPI anchors, circulating lipids can be cleaved from their lipoprotein particles and taken up into the interstitial cells via the lipid transporter FATP1 (5). These triglycerides are then able to be safely stored in lipid droplets within the adipocyte for times of a caloric shortfall.

During the fasted state, levels of circulating epinephrine rise, driving the activation of perillipin1 which in turn phosphorylates the protein Hormone Sensitive Lipase (HSL) (6). Adipose tissue also contains adipose triglycerides lipase (ATGL), a specific lipase with similar function to HSL which is activated by CGI-58 and is inhibited by perillipin 5 (7, 8). HSL and ATGL in combination drive the removal of long chain fatty acids from stored triglycerides, freeing fatty acids to circulate to other tissues. Interestingly, in the fasted state, muscle upregulates LPL, the inverse of adipose tissue, in an effort to accumulate lipids as a fuel source in the absence of glucose (9). Many of these dynamic aspects of fatty acid release are potently inhibited by insulin's action in the fed state. With such an obvious phenotype of lipid storage and distribution regulated by the anabolic hormone insulin, it is understandable that many additional aspects of adipose physiology remained unexplored (10).

Adipocytes- More than a storage vessel

For many years the view of adipose tissue was primarily that of a metabolically inert energy storage tissue. However in the mid 1990's, this view changed dramatically when it was discovered that adipocytes had the capacity to modulate both peripheral and central signaling in mammals. The finding

that adipocytes provided endocrine function redefined these cells and their pivotal role in endocrinology and energy homeostasis.

The mouse obese gene (or *ob* gene) was initially identified as a protein whose loss of function was responsible for dramatic hyperphagia and extreme obesity and diabetes in mice (11). Over the next several years, it became apparent that this gene product was sufficient to regulate food intake in living animals, as injections of recombinant protein caused dramatic decreases in food intake and body weight (12). Furthermore, as it was shown that the product of this gene was produced predominantly in the adipocyte, the adipocyte moved center stage in the field of endocrinology. Much attention was subsequently focused on this initially innocuous cell whose volume is comprised of over 90% lipid. Concurrent with the increased attention on adipose tissue was the dramatic rise in obesity in both the United States and worldwide.

Obesity Epidemic

The health and maintenance of adipose tissue has become increasingly relevant. In 2016, both Western societies as well as the developing world suffer from an unrelenting epidemic of obesity. Easy access and overconsumption of calorically dense foods, combined with a myriad of genetic and environmental factors have led to a current state where greater than 65% of US adults are obese or overweight (13). Obesity is the number one risk factor of the development of type two diabetes, a dangerous metabolic disorder which causes significant morbidity and mortality. As the trends in obesity and diabetic diagnosis continue to increase, a better understanding of the disease is required to properly manage the disease and its symptoms.

Adipose Tissue and Diabetes

Diabetes is recognized as a disease of elevated blood glucose levels. For type 2 diabetics, those with intact beta cells, peripheral insulin resistance represents a primary cause of elevated blood glucose

levels (14). Adipocytes respond to insulin by translocating a glucose transporter 4 (Glut4) to the plasma membrane which in turn prompts enhanced glucose uptake into these cells. In the case of insulin resistance, adipose tissue is no exception and insulin resistance in this tissue is a widely recognized problem . With loss of the proper responses to insulin, adipose tissue is unable to meet the metabolic demands of the system. This in turn results in elevated blood glucose levels, and and a myriad of additional metabolic problems, all stemming from the dysfunctional nature of adipose tissue during insulin resistance.

Hepatic adipose crosstalk

The liver receives a large proportion of the body's blood in two unique ways. First it receives oxygenated blood via the hepatic artery. More interestingly, however, is the blood it receives via the portal vein which arrives post capillary bed coming from both visceral adipose tissue and the intestines. This blood supply provides the liver with first line access to many nutrients, including glucose and amino acids derived from nutrient intake. Commonly observed in both the clinical and laboratory settings, those patients and rodents with obesity and prediabetes or fulminant type 2 diabetes regularly develop fatty liver disease, associated with dysregulated gluconeogenesis (15). Dysfunction within either adipose tissue or the liver can result in metabolic dysfunction in the other tissue. Our laboratory has documented this time and again showing that enhancing the function of adipose tissue improves hepatic insulin sensitivity and hepatic steatosis (16). We know from studies in our lab that primary drivers in this cross talk are protein hormones secreted by the liver and adipose tissue respectively. For example, it was shown that HFD feeding causes a decrease in adiponectin secretion, and that adiponectin has many beneficial effects on the liver, including decreasing gluconeogenesis during fed states ((17), (18)) Furthermore, our laboratory has demonstrated that adiponectin modulates the neutralization of insulin resistance causing lipids in the liver and other organs; again providing link between altered adipose function and diminished capacity for the liver to fulfill its job providing

metabolic homeostasis (19). These types of studies highlight the incredible importance of adipose tissue and provide resounding evidence that we must fully understand adipose dysfunction in order to fully understand the metabolic perturbations found in type 2 diabetes if we are to adequately treat patients.

Adipose Tissue Dysfunction

With over consumption of calorically dense foods, decreased physical activity, and a host of genetic factors, adipose tissue can very quickly become dysfunctional. This is exemplified in mouse models of high fat diet-induced obesity where in mice fed a high fat that quickly become dysfunctional and insulin resistant (20). Much of this dysfunction stems initially from the increased cell size of the adipocytes and a decrease in the ability of the vasculature to maintain adequate oxygenation, leading to hypoxia.

Hypoxic adipose tissue

The feeding of a high fat diet to rodents causes adipogenesis in the gonadal adipose depot; however in the subcutaneous depot, the sole mechanism of compensation for overnutrition is hypertrophy, or increasing in average fat cell size (21). As the adipocytes increase in size, the depot does not proportionally increase its vasculature. This is due to the inability for the hypoxic adipose to properly respond with enhanced angiogenesis (22).

During high fat diet feeding, significant hypoxia prevails in adipose tissue (23). This was also further confirmed through increased pimonidazole staining by immunohistochemistry in adipose tissue of mice fed a high fat diet, a classical marker used for the staining of hypoxic tissues. The adipose tissue of HFD fed mice is less perfused based on infusion studies using labeled microspheres. Furthermore, *in vivo* that these hypoxic conditions are associated with increased mRNA of known HIF1-α target genes. These effects are cell autonomous *in vitro,* since exposing differentiated 3T3-L1 adipocytes to hypoxic conditions resulted in comparable gene expression changes. These experiments were among the first to

demonstrate hypoxia in adipose tissue and the authors hypothesized that these changes could be physiologically detrimental.

In an effort to validate that hypoxia was truly causative in adipose tissue dysfunction, Sun et al. developed an adipose specific overexpression of VEGF-A, a protein capable of driving increased angiogenesis (24). This mouse showed dramatic improvements in overall metabolic parameters, including fed blood glucose when challenged with a HFD. Furthermore, overexpression of VEGF-A during HFD feeding ameliorated hypoxia in adipose tissue and diminished the expression of HIF1-α gene targets. Unexpectedly, these mice also demonstrated a beiging phenotype of the white subcutaneous adipose depot, though a mechanism accounting for this result was not fully explored. Overall, this data confirmed increasing the vasculature in white adipose tissue during HFD feeding is sufficient to ameliorate the observed metabolic dysfunction. Their data was further substantiated by another group using both adipose tissue-specific deletion and overexpression of VEGF-A (25). These results confirmed that the loss of VEGF-A in adipocytes during high fat diet feeding causes deleterious metabolic effects, while overexpression resulted in dramatically improved metabolic homeostasis. Importantly they demonstrated that loss of VEGF-A from adipocytes results in dramatic increases in macrophage infiltration into the adipose with concomitant exacerbation in the inflammatory state of this tissue. Although these papers demonstrated that improved vascularization was beneficial during a metabolic challenge, the question of the specific role of HIF1-α in adipose tissue, and whether its inhibition is a worthwhile target for pharmaceutical intervention during HFD challenge, remains a question under study.

To address this question, Sun et al. used the known HIF1-α inhibitor PX-478 (26). Mice fed a HFD and treated with PX478 showed a dramatic decrease in the expression of known HIF1-α targets in their adipose depots. These mice further showed a decrease in body weight gain on high fat diet and a resistance to the metabolic dysfunction that ensues. They showed dramatic improvements in glucose

tolerance and an increase in energy expenditure. Most importantly, this treatment exhibited a suppressed inflammatory response in adipose tissue upon high fat diet feeding as well as a dramatic reduction in fibrosis, a key player in HFD induced metabolic dysfunction. Overall these works demonstrate that HFD induced hypoxia is causal in its ability to increase inflammation and fibrosis in adipose tissue, and further that this stems from activation of HIF1-α and its downstream mediators.

Fibrosis

Adipose tissue hypoxia leads to metabolic dysfunction. A primary mediator of the downstream sequelae of hypoxia is HIF1-α. This transcription factor directly regulates the expression of TGF-β, a primary transcription factor in the regulation of collagen gene synthesis (27, 28). Furthermore, HIF1-α directly regulates collagen hydroxylation by driving transcription of prolyl hydroxylase, thereby increasing collagen secretion and enhancing folding (29). The primary collagens in obese adipose tissue are collagen I, III and VI, with the latter being secreted from the adipocytes themselves (30). Interestingly, much work has been done to show that adipose tissue collagen is a crucial player in depot insulin sensitivity and whole body glucose homeostasis.

To demonstrate that HIF1-α directly drives adipose fibrosis and dysfunction, Halberg et al. overexpressed a constitutively active form of HIF1-α in adipocytes (22). The authors demonstrated that adipose tissue has decreased VEGF-A and increased HIF1-α by immunohistochemistry staining in the obese state; indicating a clear disconnect in this organ between hypoxic sensing and the necessary angiogenic response. They go on to overexpress a constitutively active form of HIF1-α specifically in adipocytes, which resulted in decreased glucose tolerance and increased hepatic triglyceride levels. Finally they show that collagens I, III and VI as well as elastin are all upregulated in the adipose depots of these mice; and this is further supported by dramatically elevated trichrome staining indicating reflecting enhanced fibrosis.

In an inverse set of experiments, Khan et al demonstrated that the loss of collagen VI results in a dramatic improvement in whole body glucose homeostasis (31). After identifying collagen VI as the most abundant collagen in all adipose depots of genetically obese mice (*db/db*) they decided to test the metabolic benefits of the loss of collagen VI. They crossed the Collagen VI-alpha1 mutant mouse into the *ob/ob* background. Initially these mice displayed a decreased body weight, which normalized over time. Metabolically these mice displayed dramatic improvements in glucose tolerance, insulin sensitivity, triglyceride clearance and hepatic steatosis.

Taken together, these papers demonstrate that hypoxia-driven collagen deposition is a *bona fide* force in the observed metabolic dysfunction seen in the obese state. While enhancing hypoxic response elements increased collagen deposition and enhanced metabolic deterioration, loss of collagen resulted in dramatically improved metabolic parameters on challenge. Despite these findings, the underlying mechanisms by which collagen deposition results in metabolic dysfunction remained unexplored.

At a cellular level, Pellegrinelli et al. elegantly demonstrated that mechanical strain has effects on adipocyte biology (32). Physical deformation of adipocytes activated a number of genes including connective tissue growth factor (CTGF) in a β1 integrin dependent manner. The authors found that adipocytes placed into an obese, de-cellularized adipose tissue matrix had dramatically different expression of a number of genes including ATF4, IL6 and CCL5; and that these genes were further altered with increased deformation of the matrix. They went on to show that deformation and the obese decelluarlized matrix activates CTGF gene targets in a YAP-TEAD dependent pathway. Together these data show that rigidity in the adipocyte matrix can alter metabolic and inflammatory pathways, therefore providing a causal link between fibrotic deposition and metabolic dysfunction in white adipose tissue.

Inflammation in adipose tissue

Of all the adipokines relevant to metabolism and disease, inflammatory adipokines have taken the spotlight. During HFD feeding in rodents, or the obese state in humans, inflammatory markers in both circulation as well in adipose depots are robustly increased (33). Among those factors which play a role in inflammation and are *bona fide* adipokines secreted from adipocytes include a plethora of bioactive molecules, though herein I will limit the discussion to three key factors: TNF-α, IL-6 and MCP-1.

The role of inflammatory cytokines being causative in metabolic dysfunction during states of obesity exploded onto the metabolism and endocrinology scene in the mid 1990's. Work by Hotamisligil and Spiegelman demonstrated that TNF-α was causative in metabolic dysfunction in obese rodents. Their first paper in 1993 identified elevated TNF-α transcript in the adipose tissue of obese mice (*db/db*) and obese rats (*fa/fa*) (34). They further went on to demonstrate that TNF-α directly alters the gene expression in cultured adipocytes of many metabolically relevant genes including Glut1 and Glut4. Further, they showed that neutralization of TNF-α with an antibody in obese rats (*fa/fa*) improved glucose infusion in a euglycemic clamps. This paper highlighted the importance of inflammation in adipose tissue and identified a possible means by which to target metabolic dysfunction during obesity.

Following their initial report, Hotamisligil et al. went on to publish additional observations laying the groundwork for the field of inflammatory adipose biology. A year later, they showed that chronic exposure of adipocytes to low levels of TNF-α impairs insulin stimulated glucose uptake (35). They further showed that TNF-α blocked the ability of the insulin receptor to phosphorylate IRS-1, its primary downstream mediator. Elevated TNF-α expression is not limited to obese rodents, but can also be found in obese insulin resistant humans (36). Elevated TNF-α transcripts are seen in the adipose depots of obese females, and they further correlated the transcript levels with a variety of diabetes phenotypes, including elevated plasma insulin, glucose and triglycerides. Most importantly, they demonstrated that TNF-α decreases following weight loss, and this was to a similar extent as the decrease seen in

circulating insulin levels. This established the field of immunometabolism, and moreover, highlighted the dysregulation of adipose during obesity and the subsequent inflammatory burden that follows.

IL-6

With these initial observations demonstrating that TNF-α, a commonly known and well-understood inflammatory cytokine, is elevated during states of obesity and likely causative for the insulin resistant state associated with diabetes; the field started to look for other possible candidates for therapeutic intervention. Interleukin-6 (IL-6) had previously been identified as having potent metabolic effects on adipose tissue, however these initial studies were performed as part of a program to limit cachectic wasting during tumor growth (37). These studies found that IL-6 reduces LPL activity in adipose tissue, though this was via a TNF-α dependent pathway.

Shortly following Hotamisligil's initial studies on TNF-α, several papers demonstrated very clearly that IL-6 levels are elevated in those patients that were non-insulin dependent diabetics (38). Within a few years, others identified similar patterns in obese humans, and would further provide causal mechanisms by which IL-6 inhibits insulin signaling in cultured adipocytes (39). They showed here that IL-6 directly downregulates IRS-1 and Glut-4 transporter in cultured 3T3-L1 adipocytes in a manner very similar to that demonstrated for TNF-α. Since elevated IL-6 during obese states has been established, many groups have identified elevated IL-6 correlating with a number of metabolic perturbations related to diabetes, including increased atherosclerosis-related heart attacks and strokes (40).

Macrophages

Almost 10 years after the initial identification of elevated inflammation in adipose tissue of obese individuals, the macrophage was identified as a major driver of the process. In 2003, multiple groups simultaneously published a rather startling discovery (41, 42). First, Anthony Ferrante's group showed that in an analysis of transcripts from adipose tissue that positively correlated with body mass index in

mice, 30% of these transcripts belonged to macrophage-specific genes. They further showed by immunohistochemical staining that macrophages are an abundant cell type in the adipose, and via transplantation experiments, determined these were truly CSF-1 dependent bone marrow-derived mononuclear cells infiltrating into the adipose. Finally, they showed that macrophage infiltration into adipose tissue is seen in human adipose tissue as well, and that this correlated with BMI as well as adipocyte size, both predictors of poor metabolic health.

Another group started with the observation that diet-induced obesity due to HFD feeding leads to an upregulation of macrophage genes in white adipose tissue (42). Separating adipose tissue into stromal vascular and adipocyte fractions, they showed an abundance of inflammatory message residing in the stromal vascular fraction (SVF) containing macrophages. The vast majority of TNF-α mRNA resides in this SVF. Finally, they went on to show that the PPAR-γ agonist rosiglitazone is capable of counteracting this phenomenon, resulting in a significant decrease in MCP-1, F4/80 and CD68 message in adipose tissue.

Taken together, these papers set an entirely new precedent in the field of adipose biology. Firstly, macrophages are accumulating in the adipose of genetic- and diet-induced obesity in both rodents and humans. Even more importantly than this, they appeared to be functionally contributing to the inflammation seen in these states; inflammation which had been validated as potentially causal with respect to its ability to inhibit insulin signaling and perpetuate the diabetic state. With these initial findings, the field of macrophage biology in adipose tissue had seen its birth.

Very interestingly, shortly before these papers were published, another group identified MCP-1 as an insulin responsive protein in obese mice derived from adipocytes (43). They showed this responsiveness both *in vivo* (3T3-L1 cells) and *in vitro*, and demonstrated that the level of this protein directly correlates with obesity in rodents. Although they demonstrated that MCP-1 altered the function of adipocytes,

they did not directly connect it to its assumed function as a chemoattractant directing macrophages to adipose tissue.

In 2008, Patsouris et al. demonstrated that depletion of CD11c+ cells from rodents can normalize insulin resistance in the obese setting (44). Previously another group had identified CD11c+ infiltrates in adipose tissue as being highly specific to obesity and producing an enormous amount of inflammatory molecules including TNF-α and IL-6 (45). With the use of a CD11c targeted diphtheria toxin model, Patsouris convincingly showed that depletion of these cells dramatically improved insulin sensitivity and glucose tolerance while decreasing "crown-like structures" and inflammatory cytokine production within the white adipose depots. Overall, this data points to inflammation in adipose tissue as being highly detrimental to the metabolic state of the organism. However, more recently our group has challenged this hypothesis.

Using several models of attenuated inflammatory response in mouse adipose tissue, Asterholm et al. demonstrated that adipocyte inflammation is required for adequate response to high fat diet in rodents (46). To attenuate inflammation, we used an adipocyte specific overexpression of a dominant negative TNF-α or an overexpression of the viral protein called RID, which attenuates the effects of signaling via TNF-α, TLR4 and IL1-β. Challenging these models with a HFD showed decreased weight gain with a paradoxical glucose-intolerant phenotype despite similar body weights and glucose tolerance on chow diet. This paper goes on to demonstrate that inhibition of adipose depot inflammation decreases adipogenesis, resulting in increased hepatic triglyceride content due to loss of this depot. Overall, this work challenged the dogmatic view of adipose inflammation as exclusively harmful following high fat diet. While chronic inflammatory insults result in decreased insulin sensitivity and glucose tolerance, some inflammation appears to be necessary for proper formation of adipose depots.

Other Adipkonines

Over the subsequent couple of years, it became clear that the adipocyte secreted a wide variety of bioactive molecules, many with far reaching effects. With later discoveries of many additional secreted proteins from the adipocyte, the term adipokine was coined. These proteins can be separated into 2 classes, those highly specific to the adipocyte, of which leptin is an example, and those which are secreted from both adipocytes as well as many other cells throughout the body.

FABP4, also known as aP2, is a highly abundant protein adipose tissue. The first genetic knockout of aP2 demonstrated significant protection from high fat diet induced obesity (47). These mice gained equal amounts of body weight, but showed dramatically less hyperinsulinemia and marked inhibition of HFD induced inflammation in adipose tissue as demonstrated by decreased TNF-α transcript in this depot. However, later reports cast doubt on these stark phenotypes, demonstrating similar levels of hyperinsulinemia and inflammation in adipose tissue (48). These authors did found however enhanced conversion of glucose into fatty acids. They ascribed the differences between their work and others to the enhanced expression of KFABP (Keratinocyte fatty acid binding protein), a protein with a compensatory upregulation upon the loss of aP2/FABP4 from adipocytes.

Another highly adipose specific protein, at least for mice and other rodents, is resistin. Initially identified as a protein which was downregulated in adipose tissue in response to PPAR-γ agonists, resistin was thought to mediate resistance to insulin (as the name suggests) (49). Work from this first paper showed elevation of resistin in serum and in adipose tissue upon HFD feeding, and neutralization of the protein correlated with improved hyperglycemia and insulin resistance. More recent work suggests a role for resistin to modulate the activity of glucose dependent insulinotropic peptide which in turn regulates lipoprotein lipase activity, a well-known mediator of lipid uptake (50, 51). However the relevance of resistin has recently been called into question as the expression patters are dramatically different between humans and rodents; citing that in humans resistin is primarily expressed in mononuclear

white blood cells (52). However, our group has demonstrated that resistin can cause leptin resistance centrally in the hypothalamus (53).

A plethora of other adipokines are less specific to adipose tissue than these proteins, but are none the less important adipose secreted factors. Recently the adipose derived protein adipsin has garnered much attention for its capacity to enhance β cell function during diabetes (54). Classically known as compliment Factor D, this protein is known for its requirement in the alternative compliment activation pathway and the clearance of pathogens (55). Of interest in metabolism, this protein's circulating levels decrease dramatically during HFD feeding. Adipsin knockout mice were shown to be glucose intolerant as a result of impaired β cell function.

Another emerging adipokine whose expression is not restricted solely to the adipocyte is chemerin. Approximately 10 years ago, 2 groups identified that this protein, already known to be expressed in other tissues, was expressed in 3T3-L1 differentiated adipocytes, and that the levels of this protein correlated with a number of metabolic parameters ((56),(57)). Others have gone on to show that chemerin levels are decreased with exercise, and that overexpression of this protein causes impaired insulin sensitivity in muscle without affecting other tissues. (58) (59). More recent published data has suggested a mechanism by which chemerin functions through GPR1 (G-protein coupled receptor 1), as it binds this receptor with high affinity, though the knockout of GPR1 led to dichotomous phenotypes, including reduced insulin levels but enhanced pyruvate-induced plasma glucose levels. Therefore, the mechanism and relevance of chemerin to metabolic disease remain to be better understood (60).

Adiponectin

Adiponectin is a protein produced and secreted exclusively by adipocytes. First identified in 1995, it has become one of the most common biomarkers used in the evaluation of metabolic disorders. Its use as a biomarker stems from the incredibly tight correlations between increasing insulin resistance and

decreasing circulating adiponectin in serum (61). Mechanistically, we have only recently begun to understand the mechanisms by which adiponectin exerts its metabolically beneficial effects. Adiponectin for many years has been demonstrated to be protective *in vivo* for a wide variety of insults, including HFD-induced obesity, heart damage as well as atherosclerosis (62, 63).

Long thought to act via its receptors to activate AMPK, recently our group has shown that adiponectin acts to modulate ceramide levels intracellularly (19). The cleavage of these lipid species and the further generation of sphingosine-1-phosphate result in activation of AMPK and the downstream beneficial effects (19). Even more recently, a group has identified that adiponectin engaging its receptors results in neutral ceramidase being recruited to the cell surface (64). This paper sheds light to a long unanswered question of whether the adiponectin receptors themselves contained intrinsic ceramidase activity, or whether they recruited a ceramidase, with the later now being the most likely scenario.

Premise and Hypothesis

High fat diet feeding is known to cause many alterations to adipose tissue resulting in the reduced capacity for this tissue to perform its function. Hypertrophic expansion of adipose tissue resulting in hypoxic conditions initiates a fibrotic and inflammatory cascade. This maladaptive response and in appropriate tissue remodeling are responsible for local and systemic alterations in glucose and lipid handling as well as overall insulin sensitivity.

I hypothesized that another initiating event in this deleterious cascade is decreased insulin sensitivity. I further postulate that maintenance of insulin signaling during HFD feeding in adipose tissue will reduce inflammation, increase secretion of major adipokines, and will improve whole body metabolism including enhanced insulin sensitivity. Over the course of my thesis, I have tested this hypothesis at multiple levels.

References

1. Chang L, Chiang SH, Saltiel AR. Insulin signaling and the regulation of glucose transport. Molecular medicine. 2004;10(7-12):65-71. Epub 2005/11/25. doi: 10.2119/2005-00029.Saltiel. PubMed PMID: 16307172; PubMed Central PMCID: PMC1431367.
2. Dimitriadis G, Mitrou P, Lambadiari V, Maratou E, Raptis SA. Insulin effects in muscle and adipose tissue. Diabetes research and clinical practice. 2011;93 Suppl 1:S52-9. Epub 2011/09/01. doi: 10.1016/S0168-8227(11)70014-6. PubMed PMID: 21864752.
3. Jayakumar A, Chirala SS, Chinault AC, Baldini A, Abu-Elheiga L, Wakil SJ. Isolation and chromosomal mapping of genomic clones encoding the human fatty acid synthase gene. Genomics. 1994;23(2):420-4. Epub 1994/09/15. doi: 10.1006/geno.1994.1518. PubMed PMID: 7835891.
4. Takeuchi K, Reue K. Biochemistry, physiology, and genetics of GPAT, AGPAT, and lipin enzymes in triglyceride synthesis. American journal of physiology Endocrinology and metabolism. 2009;296(6):E1195-209. Epub 2009/04/02. doi: 10.1152/ajpendo.90958.2008. PubMed PMID: 19336658; PubMed Central PMCID: PMC2692402.
5. Wu Q, Ortegon AM, Tsang B, Doege H, Feingold KR, Stahl A. FATP1 is an insulin-sensitive fatty acid transporter involved in diet-induced obesity. Molecular and cellular biology. 2006;26(9):3455-67. Epub 2006/04/14. doi: 10.1128/MCB.26.9.3455-3467.2006. PubMed PMID: 16611988; PubMed Central PMCID: PMC1447434.
6. Kraemer FB, Shen WJ. Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. Journal of lipid research. 2002;43(10):1585-94. Epub 2002/10/05. PubMed PMID: 12364542.
7. Lass A, Zimmermann R, Haemmerle G, Riederer M, Schoiswohl G, Schweiger M, et al. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. Cell metabolism. 2006;3(5):309-19. Epub 2006/05/09. doi: 10.1016/j.cmet.2006.03.005. PubMed PMID: 16679289.
8. Wang H, Bell M, Sreenivasan U, Hu H, Liu J, Dalen K, et al. Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. The Journal of biological chemistry. 2011;286(18):15707-15. Epub 2011/03/12. doi: 10.1074/jbc.M110.207779. PubMed PMID: 21393244; PubMed Central PMCID: PMC3091179.
9. Fielding BA, Frayn KN. Lipoprotein lipase and the disposition of dietary fatty acids. The British journal of nutrition. 1998;80(6):495-502. Epub 1999/04/22. PubMed PMID: 10211047.
10. Yu YH, Ginsberg HN. Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. Circulation research. 2005;96(10):1042-52. Epub 2005/05/28. doi: 10.1161/01.RES.0000165803.47776.38. PubMed PMID: 15920027.
11. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994;372(6505):425-32. Epub 1994/12/01. doi: 10.1038/372425a0. PubMed PMID: 7984236.
12. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science. 1995;269(5223):546-9. Epub 1995/07/28. PubMed PMID: 7624778.
13. Kumar N, Choudhary NS. Treating morbid obesity in cirrhosis: A quest of holy grail. World journal of hepatology. 2015;7(28):2819-28. Epub 2015/12/17. doi: 10.4254/wjh.v7.i28.2819. PubMed PMID: 26668693; PubMed Central PMCID: PMC4670953.

14. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes care. 2009;32 Suppl 2:S157-63. Epub 2009/11/13. doi: 10.2337/dc09-S302. PubMed PMID: 19875544; PubMed Central PMCID: PMC2811436.
15. Bhatt HB, Smith RJ. Fatty liver disease in diabetes mellitus. Hepatobiliary surgery and nutrition. 2015;4(2):101-8. Epub 2015/05/26. doi: 10.3978/j.issn.2304-3881.2015.01.03. PubMed PMID: 26005676; PubMed Central PMCID: PMC4405411.
16. Morley TS, Xia JY, Scherer PE. Selective enhancement of insulin sensitivity in the mature adipocyte is sufficient for systemic metabolic improvements. Nature communications. 2015;6:7906. Epub 2015/08/06. doi: 10.1038/ncomms8906. PubMed PMID: 26243466; PubMed Central PMCID: PMC4527086.
17. Miller RA, Chu Q, Le Lay J, Scherer PE, Ahima RS, Kaestner KH, et al. Adiponectin suppresses gluconeogenic gene expression in mouse hepatocytes independent of LKB1-AMPK signaling. The Journal of clinical investigation. 2011;121(6):2518-28. Epub 2011/05/25. doi: 10.1172/JCI45942. PubMed PMID: 21606593; PubMed Central PMCID: PMC3104763.
18. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nature medicine. 2001;7(8):947-53. Epub 2001/08/02. doi: 10.1038/90992. PubMed PMID: 11479628.
19. Holland WL, Miller RA, Wang ZV, Sun K, Barth BM, Bui HH, et al. Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. Nature medicine. 2011;17(1):55-63. Epub 2010/12/28. doi: 10.1038/nm.2277. PubMed PMID: 21186369; PubMed Central PMCID: PMC3134999.
20. Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. The Journal of clinical investigation. 2011;121(6):2094-101. Epub 2011/06/03. doi: 10.1172/JCI45887. PubMed PMID: 21633177; PubMed Central PMCID: PMC3104761.
21. Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nature medicine. 2013;19(10):1338-44. Epub 2013/09/03. doi: 10.1038/nm.3324. PubMed PMID: 23995282; PubMed Central PMCID: PMC4075943.
22. Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, et al. Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. Molecular and cellular biology. 2009;29(16):4467-83. Epub 2009/06/24. doi: 10.1128/MCB.00192-09. PubMed PMID: 19546236; PubMed Central PMCID: PMC2725728.
23. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes. 2007;56(4):901-11. Epub 2007/03/31. doi: 10.2337/db06-0911. PubMed PMID: 17395738.
24. Sun K, Wernstedt Asterholm I, Kusminski CM, Bueno AC, Wang ZV, Pollard JW, et al. Dichotomous effects of VEGF-A on adipose tissue dysfunction. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(15):5874-9. Epub 2012/03/28. doi: 10.1073/pnas.1200447109. PubMed PMID: 22451920; PubMed Central PMCID: PMC3326476.
25. Sung HK, Doh KO, Son JE, Park JG, Bae Y, Choi S, et al. Adipose vascular endothelial growth factor regulates metabolic homeostasis through angiogenesis. Cell metabolism. 2013;17(1):61-72. Epub 2013/01/15. doi: 10.1016/j.cmet.2012.12.010. PubMed PMID: 23312284.
26. Sun K, Halberg N, Khan M, Magalang UJ, Scherer PE. Selective inhibition of hypoxia-inducible factor 1alpha ameliorates adipose tissue dysfunction. Molecular and cellular biology. 2013;33(5):904-17. Epub 2012/12/20. doi: 10.1128/MCB.00951-12. PubMed PMID: 23249949; PubMed Central PMCID: PMC3623075.
27. Schaffer L, Scheid A, Spielmann P, Breymann C, Zimmermann R, Meuli M, et al. Oxygen-regulated expression of TGF-beta 3, a growth factor involved in trophoblast differentiation. Placenta. 2003;24(10):941-50. Epub 2003/10/29. PubMed PMID: 14580376.

28. Bhogal RK, Stoica CM, McGaha TL, Bona CA. Molecular aspects of regulation of collagen gene expression in fibrosis. Journal of clinical immunology. 2005;25(6):592-603. Epub 2005/12/29. doi: 10.1007/s10875-005-7827-3. PubMed PMID: 16380822.
29. Bentovim L, Amarilio R, Zelzer E. HIF1alpha is a central regulator of collagen hydroxylation and secretion under hypoxia during bone development. Development. 2012;139(23):4473-83. Epub 2012/10/26. doi: 10.1242/dev.083881. PubMed PMID: 23095889.
30. Divoux A, Tordjman J, Lacasa D, Veyrie N, Hugol D, Aissat A, et al. Fibrosis in human adipose tissue: composition, distribution, and link with lipid metabolism and fat mass loss. Diabetes. 2010;59(11):2817-25. Epub 2010/08/18. doi: 10.2337/db10-0585. PubMed PMID: 20713683; PubMed Central PMCID: PMC2963540.
31. Khan T, Muise ES, Iyengar P, Wang ZV, Chandalia M, Abate N, et al. Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. Molecular and cellular biology. 2009;29(6):1575-91. Epub 2008/12/31. doi: 10.1128/MCB.01300-08. PubMed PMID: 19114551; PubMed Central PMCID: PMC2648231.
32. Pellegrinelli V, Heuvingh J, du Roure O, Rouault C, Devulder A, Klein C, et al. Human adipocyte function is impacted by mechanical cues. The Journal of pathology. 2014;233(2):183-95. Epub 2014/03/14. doi: 10.1002/path.4347. PubMed PMID: 24623048.
33. Bahceci M, Gokalp D, Bahceci S, Tuzcu A, Atmaca S, Arikan S. The correlation between adiposity and adiponectin, tumor necrosis factor alpha, interleukin-6 and high sensitivity C-reactive protein levels. Is adipocyte size associated with inflammation in adults? Journal of endocrinological investigation. 2007;30(3):210-4. Epub 2007/05/17. PubMed PMID: 17505154.
34. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259(5091):87-91. Epub 1993/01/01. PubMed PMID: 7678183.
35. Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(11):4854-8. Epub 1994/05/24. PubMed PMID: 8197147; PubMed Central PMCID: PMC43887.
36. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. The Journal of clinical investigation. 1995;95(5):2409-15. Epub 1995/05/01. doi: 10.1172/JCI117936. PubMed PMID: 7738205; PubMed Central PMCID: PMC295872.
37. Greenberg AS, Nordan RP, McIntosh J, Calvo JC, Scow RO, Jablons D. Interleukin 6 reduces lipoprotein lipase activity in adipose tissue of mice in vivo and in 3T3-L1 adipocytes: a possible role for interleukin 6 in cancer cachexia. Cancer research. 1992;52(15):4113-6. Epub 1992/08/01. PubMed PMID: 1638523.
38. Pickup JC, Mattock MB, Chusney GD, Burt D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia. 1997;40(11):1286-92. Epub 1997/12/06. doi: 10.1007/s001250050822. PubMed PMID: 9389420.
39. Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. The Journal of biological chemistry. 2003;278(46):45777-84. Epub 2003/09/04. doi: 10.1074/jbc.M301977200. PubMed PMID: 12952969.
40. Hartman J, Frishman WH. Inflammation and atherosclerosis: a review of the role of interleukin-6 in the development of atherosclerosis and the potential for targeted drug therapy. Cardiology in review. 2014;22(3):147-51. Epub 2014/03/13. doi: 10.1097/CRD.0000000000000021. PubMed PMID: 24618929.
41. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. The Journal of clinical investigation.

2003;112(12):1796-808. Epub 2003/12/18. doi: 10.1172/JCI19246. PubMed PMID: 14679176; PubMed Central PMCID: PMC296995.
42. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. The Journal of clinical investigation. 2003;112(12):1821-30. Epub 2003/12/18. doi: 10.1172/JCI19451. PubMed PMID: 14679177; PubMed Central PMCID: PMC296998.
43. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(12):7265-70. Epub 2003/05/21. doi: 10.1073/pnas.1133870100. PubMed PMID: 12756299; PubMed Central PMCID: PMC165864.
44. Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM, Neels JG. Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. Cell metabolism. 2008;8(4):301-9. Epub 2008/10/09. doi: 10.1016/j.cmet.2008.08.015. PubMed PMID: 18840360; PubMed Central PMCID: PMC2630775.
45. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. The Journal of clinical investigation. 2007;117(1):175-84. Epub 2007/01/04. doi: 10.1172/JCI29881. PubMed PMID: 17200717; PubMed Central PMCID: PMC1716210.
46. Wernstedt Asterholm I, Tao C, Morley TS, Wang QA, Delgado-Lopez F, Wang ZV, et al. Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. Cell metabolism. 2014;20(1):103-18. Epub 2014/06/17. doi: 10.1016/j.cmet.2014.05.005. PubMed PMID: 24930973; PubMed Central PMCID: PMC4079756.
47. Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE, Spiegelman BM. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. Science. 1996;274(5291):1377-9. Epub 1996/11/22. PubMed PMID: 8910278.
48. Shaughnessy S, Smith ER, Kodukula S, Storch J, Fried SK. Adipocyte metabolism in adipocyte fatty acid binding protein knockout mice (aP2-/-) after short-term high-fat feeding: functional compensation by the keratinocyte [correction of keritinocyte] fatty acid binding protein. Diabetes. 2000;49(6):904-11. Epub 2000/06/24. PubMed PMID: 10866041.
49. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, et al. The hormone resistin links obesity to diabetes. Nature. 2001;409(6818):307-12. Epub 2001/02/24. doi: 10.1038/35053000. PubMed PMID: 11201732.
50. Kim SJ, Nian C, McIntosh CH. Resistin is a key mediator of glucose-dependent insulinotropic polypeptide (GIP) stimulation of lipoprotein lipase (LPL) activity in adipocytes. The Journal of biological chemistry. 2007;282(47):34139-47. Epub 2007/09/25. doi: 10.1074/jbc.M704896200. PubMed PMID: 17890220.
51. Kim SJ, Nian C, McIntosh CH. Resistin knockout mice exhibit impaired adipocyte glucose-dependent insulinotropic polypeptide receptor (GIPR) expression. Diabetes. 2013;62(2):471-7. Epub 2012/09/25. doi: 10.2337/db12-0257. PubMed PMID: 23002036; PubMed Central PMCID: PMC3554369.
52. Huang X, Yang Z. Resistin's, obesity and insulin resistance: the continuing disconnect between rodents and humans. Journal of endocrinological investigation. 2016;39(6):607-15. Epub 2015/12/15. doi: 10.1007/s40618-015-0408-2. PubMed PMID: 26662574.
53. Asterholm IW, Rutkowski JM, Fujikawa T, Cho YR, Fukuda M, Tao C, et al. Elevated resistin levels induce central leptin resistance and increased atherosclerotic progression in mice. Diabetologia. 2014;57(6):1209-18. Epub 2014/03/14. doi: 10.1007/s00125-014-3210-3. PubMed PMID: 24623101; PubMed Central PMCID: PMC4106234.
54. Lo JC, Ljubicic S, Leibiger B, Kern M, Leibiger IB, Moede T, et al. Adipsin is an adipokine that improves beta cell function in diabetes. Cell. 2014;158(1):41-53. Epub 2014/07/06. doi: 10.1016/j.cell.2014.06.005. PubMed PMID: 24995977; PubMed Central PMCID: PMC4128197.

55. Brade V, Bentley C, Kossorotow A, Bitter-Suermann D. Activation of factor B and C3 in guinea pig serum by zymosan or by activated properdin depends on the presence of factor D. Monographs in allergy. 1977;12:83-5. Epub 1977/01/01. PubMed PMID: 917024.
56. Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, et al. Chemerin is a novel adipokine associated with obesity and metabolic syndrome. Endocrinology. 2007;148(10):4687-94. Epub 2007/07/21. doi: 10.1210/en.2007-0175. PubMed PMID: 17640997.
57. Goralski KB, McCarthy TC, Hanniman EA, Zabel BA, Butcher EC, Parlee SD, et al. Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. The Journal of biological chemistry. 2007;282(38):28175-88. Epub 2007/07/20. doi: 10.1074/jbc.M700793200. PubMed PMID: 17635925.
58. Malin SK, Navaneethan SD, Mulya A, Huang H, Kirwan JP. Exercise-induced lowering of chemerin is associated with reduced cardiometabolic risk and glucose-stimulated insulin secretion in older adults. The journal of nutrition, health & aging. 2014;18(6):608-15. Epub 2014/06/21. doi: 10.1007/s12603-014-0459-7. PubMed PMID: 24950152; PubMed Central PMCID: PMC4697740.
59. Becker M, Rabe K, Lebherz C, Zugwurst J, Goke B, Parhofer KG, et al. Expression of human chemerin induces insulin resistance in the skeletal muscle but does not affect weight, lipid levels, and atherosclerosis in LDL receptor knockout mice on high-fat diet. Diabetes. 2010;59(11):2898-903. Epub 2010/08/21. doi: 10.2337/db10-0362. PubMed PMID: 20724582; PubMed Central PMCID: PMC2963549.
60. Rourke JL, Muruganandan S, Dranse HJ, McMullen NM, Sinal CJ. Gpr1 is an active chemerin receptor influencing glucose homeostasis in obese mice. The Journal of endocrinology. 2014;222(2):201-15. Epub 2014/06/05. doi: 10.1530/JOE-14-0069. PubMed PMID: 24895415.
61. Lihn AS, Pedersen SB, Richelsen B. Adiponectin: action, regulation and association to insulin sensitivity. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2005;6(1):13-21. Epub 2005/01/19. doi: 10.1111/j.1467-789X.2005.00159.x. PubMed PMID: 15655035.
62. Guo R, Zhang Y, Turdi S, Ren J. Adiponectin knockout accentuates high fat diet-induced obesity and cardiac dysfunction: role of autophagy. Biochimica et biophysica acta. 2013;1832(8):1136-48. Epub 2013/03/26. doi: 10.1016/j.bbadis.2013.03.013. PubMed PMID: 23524376; PubMed Central PMCID: PMC3796200.
63. Asano T, Watanabe K, Kubota N, Gunji T, Omata M, Kadowaki T, et al. Adiponectin knockout mice on high fat diet develop fibrosing steatohepatitis. Journal of gastroenterology and hepatology. 2009;24(10):1669-76. Epub 2009/10/01. doi: 10.1111/j.1440-1746.2009.06039.x. PubMed PMID: 19788607.
64. Wang Y, Wang X, Lau WB, Yuan Y, Booth D, Li JJ, et al. Adiponectin inhibits tumor necrosis factor-alpha-induced vascular inflammatory response via caveolin-mediated ceramidase recruitment and activation. Circulation research. 2014;114(5):792-805. Epub 2014/01/09. doi: 10.1161/CIRCRESAHA.114.302439. PubMed PMID: 24397980; PubMed Central PMCID: PMC3961763.

Chapter 2

Selective Enhancement of Insulin Sensitivity in the Mature Adipocyte is Sufficient for Systemic Metabolic Improvements

(The following is part of a published manuscript PMID:26243466)

Thomas S. Morley[1], Jonathan Y. Xia[1] and Philipp E. Scherer[1,2†]

[1]Touchstone Diabetes Center, Department of Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, Texas 75390-8549.

[2]Department of Cell Biology, The University of Texas Southwestern Medical Center, Dallas, Texas 75390-8549

[*]Correspondence should be addressed to:
Philipp E. Scherer
Touchstone Diabetes Center
Department of Internal Medicine
University of Texas Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX, 75390-8549, USA
E-mail: Philipp.Scherer@utsouthwestern.edu
Tel: 214-648-8715
Fax: 214-648-8720

Running title: *Adipocyte-Specific PTEN Elimination*

Abstract

Dysfunctional adipose tissue represents a hallmark of type 2 diabetes and systemic insulin resistance, characterized by fibrotic deposition of collagens and increased immune cell infiltration within the depots. We wanted to assess the role of enhanced insulin signaling specifically in mature adipocytes. To address this, we generated an inducible model of loss of function of the protein *phosphatase and tensin homolog* (PTEN), a phosphatase critically involved in turning off the insulin signal transduction cascade in a cell. These mice gained more weight on chow diet, short-term and long-term high fat diet exposure. Despite the increase in weight, they retained enhanced insulin sensitivity, improvements in oral glucose tolerance tests, reduced adipose tissue inflammation and maintained elevated adiponectin levels. These improvements also prompted reduced hepatic steatosis and enhanced hepatic insulin sensitivity. Prolonging insulin action selectively in the mature adipocyte is therefore sufficient to maintain normal systemic metabolic homeostasis.

Adipose tissue is an advanced endocrine organ used for the long-term storage of energy dense lipids (1). Insulin is primarily responsible for the enhanced uptake of glucose into tissues such as muscle and adipose tissue, suppressing glucagon release from the pancreatic α cells and concurrently suppressing glucose production and release from hepatocytes. Failure to properly respond to insulin in these respective tissues as a result of insulin resistance triggers elevated glucagon levels and elevated blood glucose levels in peripheral tissues. In the long term, this leads to glucose intolerance and ultimately, type 2 diabetes. While we appreciate the essential nature of the adipocyte as an effective storage cell for excess triglycerides with potent protective action against lipotoxic effects associated with high fat diet (HFD) exposure, the systemic consequences of prolonged insulin signaling in the mature adipocyte are not well understood. A loss of function of the insulin receptor in adipocytes, the "FIRKO" mouse, displays a lean phenotype and protection against HFD-induced glucose intolerance and lowered fasting triglycerides (2). A number of additional components of the different branches of the insulin receptor signal transduction cascade have been eliminated from the adipocyte. Adipocyte-specific deletion of Grb10, for instance, leads to inhibition of lipolysis and reduced thermogenesis (3). Adipocyte-specific loss of Glut4, the insulin-responsive glucose transporter, showed a markedly different phenotype (4). Using the ap2-CRE mouse to target the Glut4 gene in adipocytes, Able and colleagues demonstrated significant glucose intolerance and insulin resistance, while adipose mass was preserved. Furthermore, insulin resistance developed in the liver and muscle, secondary to adipose dysfunction. These two observations reflect a dichotomous response to "insulin resistance" in adipose tissue. With the loss of the receptor, we see improvements in systemic glucose tolerance, whereas the loss of a key effector of insulin action, Glut4, results in marked glucose intolerance.

Of interest in this context is the specific role of insulin sensitivity of adipose tissue in orchestrating the synthesis and release of adiponectin. Adiponectin, first identified in 1995, has

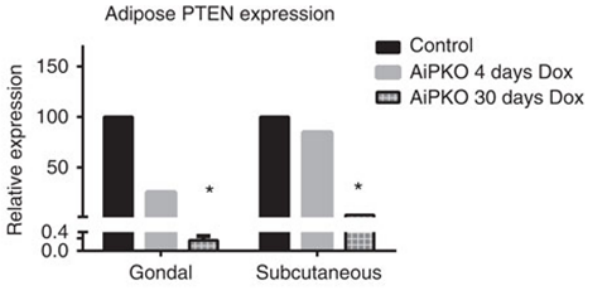
potent insulin sensitizing properties (5). Indeed, in both humans and mice, adiponectin levels strongly correlate with insulin sensitivity and are generally inversely correlated with BMI (6). Although these associations are strong, some of the most insulin resistant adipose tissues paradoxically produces extremely high levels of adiponectin. In a clinical study, Semple and colleagues identified a cohort of patients with anti-insulin receptor antibodies that render them extremely insulin resistant; surprisingly, these individuals had dramatically increased adiponectin levels in circulation (7). Furthermore, mice harboring a knockout of the insulin receptor in adipocytes had elevated intra-adipose tissue levels of adiponectin (2). In contrast, the vast majority of other models intervening with the canonical insulin signal transduction cascade display lower adiponectin levels. Therefore, with respect to the impact of insulin signaling on adiponectin production, we observe dichotomous consequences, depending on where within the insulin signal transduction cascade the system is tampered with.

In an effort to mechanistically better understand the impact of insulin signaling on adiponectin secretion, we have generated mice that allow us to manipulate insulin sensitivity in adipocytes at the post-receptor level. The phosphatase PTEN (phosphatase and tensin homolog) is a known negative regulator of insulin signaling (8). We have generated a model which allows us to inducibly delete the PTEN gene specifically in mature adipocytes, thereby greatly prolonging insulin action and enhancing the insulin sensitivity, while avoiding any developmental issues associated with the loss of function of this important phosphatase. Using an adiponectin promoter-driven rtTA transgenic mouse, crossed with the tetracycline response element driven cre-recombinase transgenic mouse (TRE-CRE) in the background of mice carrying a PTEN locus flanked by loxP sites, we can inducibly eliminate PTEN from mature adipocytes upon administration of doxycycline in the feed. This transgenic system has previously been used to track mature adipocytes during embryonic and adult life, and demonstrated to be highly specific and sensitive (9). Using this model, we can determine the effects of enhanced insulin signaling

on adipocytes in adult mice, and can further delineate the impact of prolonged high-level insulin signaling on modulating adiponectin levels in serum and the subsequent impact on whole body metabolism.

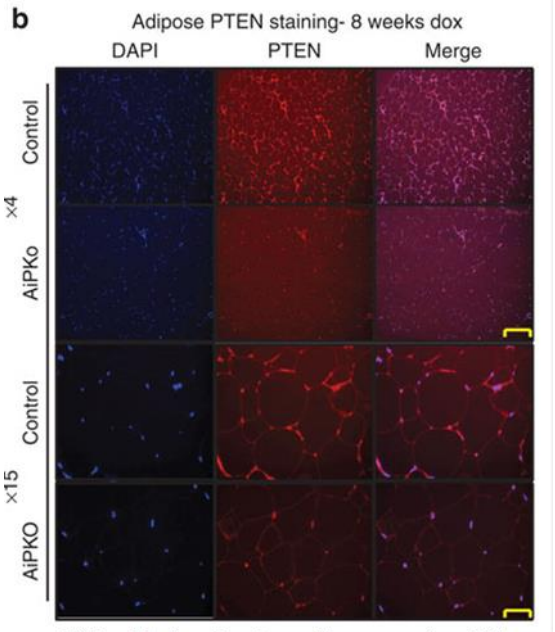
Results

Inducible, adipocyte-specific elimination of PTEN



1A) Quantitative PCR results on a floated adipocyte fraction showing PTEN gene expression from mice fed doxycycline (600mg/kg) diet for 4 or 30 days (N=2 for each bar).

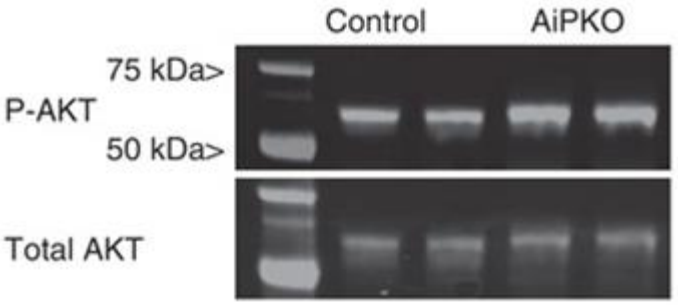
To determine whether the adipocyte-specific inducible PTEN knockout (referred to as AiPKO) mouse is effectively eliminating PTEN expression, AiPKO and control mice were placed on doxycycline diet (600mg/kg) for a period of 4 or 30 days. Subcutaneous and gonadal adipose depots were isolated from these mice, fat pads were digested with collagenase and adipocytes were separated from the stromal vascular contents by floatation. Isolated adipocytes were subjected to RNA extraction, and cDNA synthesis. RT-qPCR analysis on this cDNA confirmed that after 30 days of exposure to doxycycline, PTEN mRNA expression in adipocytes from both subcutaneous and gonadal depots is decreased by over 98% (**Fig. 1A**). To further confirm these results, adipose tissue was extracted from mice fed doxycycline diet for 10 weeks. Analysis of gonadal adipose tissue by immunofluorescence microscopy confirms widespread loss of PTEN protein from AiPKO adipocytes, while the PTEN signal clearly remained stable in the control adipocytes (**Fig. 1B**).



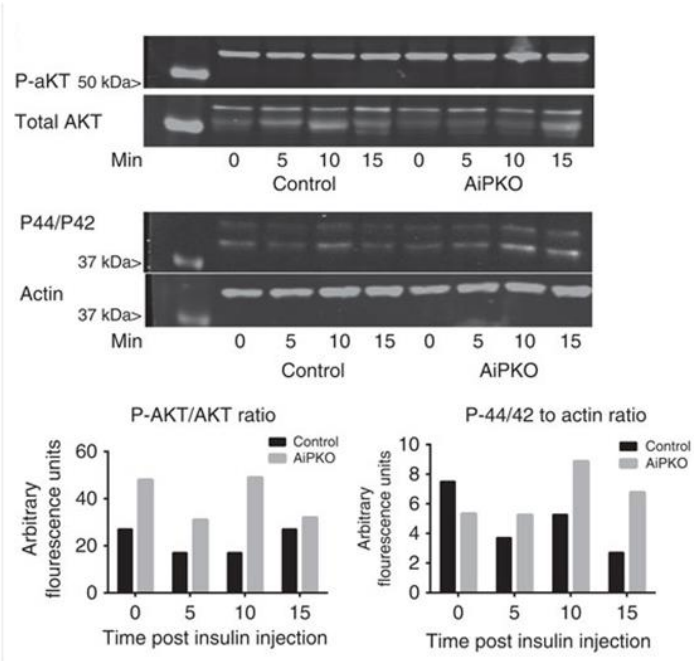
1B) Gonadal adipose tissue immunofluorescence using a PTEN antibody (Abcam 32199) with DAPI counter stain to show nuclei.

As PTEN is a known phosphatase of PiP_3, a key second messenger in the insulin signaling transduction cascade and an activator of AKT, we wished to determine if the loss of PTEN had functional consequences in a cell autonomous manner. Adipocytes were isolated from the

subcutaneous adipose depot via collagenase digestion and floatation from AiPKO or control mice after 6 weeks of doxycycline chow diet (600 mg/kg) following a 5 hour fast. These adipocytes were stimulated with 1nM insulin for 15 minutes, at which time the cells were lysed and proteins extracted for Western blot analysis. The AiPKO adipocytes demonstrated enhanced activation of the insulin signaling pathway as judged by increased phosphorylation of AKT (**Fig. 1C**). To confirm this result in-vivo, both control and AiPKO mice were placed on a doxycycline diet (600mg/kg). After 4 weeks, mice were fasted for 4 hours then I.P. injected with insulin (0.5 IU/kg) and sacrificed at 0, 5, 10 or 15 minutes. The adipose tissue from these mice was harvested; proteins extracted in TNET buffer containing protease and phosphatase inhibitors, and then subjected to western blot analysis. This analysis demonstrated enhanced AKT phosphorylation at all time points, as well as enhanced P-42/P-44 activation indicating enhanced insulin signaling through both pathways of the insulin receptor (**Fig1D)**.



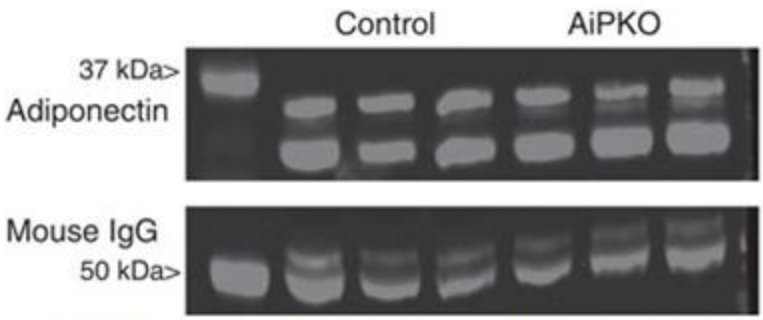
1C). Phospho-AKT, total AKT and actin for isolated adipocytes stimulated with Insulin (1nM) (N=2 for each group)



1D Western blot of protein extracts from gonadal adipose tissue harvested from mice stimulated with insulin for the indicated amount of time. Staining was then performed against phospho-AKT (S473), total AKT, p44/42 or total actin as indicated and then quantified using a Licor Imager. (1 mouse for each group (Control or AiPKO) at each time point, 0, 5, 10 or 15 minutes)

To determine whether the observed enhanced insulin signaling in adipose tissue had functional implications for adiponectin secretion, tail vein blood was drawn and Western blotting was performed on serum of mice that had been fed doxycycline containing chow diet for 4 weeks. Despite loss of PTEN

from the adipocytes, the blot revealed no significant changes in circulating adiponectin levels in the AiPKO mice compared to controls (**Fig. 1E**). At the time of blood collection, we noted that the AiPKO mice had gained more weight than the control mice over the course of the 4 weeks of doxycycline administration, though these differences were statistically not significant (data not shown). Furthermore, during blood collection, serum glucose was measured. AiPKO mice showed dramatically lower circulating glucose levels to a degree that these mice suffer from hypoglycemia (**Fig. 1F)**. To determine if this lower glucose was pertinent to overall glucose homeostasis, an OGTT was performed on the mice. The AiPKO mice demonstrated significantly better glucose handling compared to the control mice during this challenge (**Fig. 1G)**. These phenotypic changes demonstrating increased weight gain with concomitant lowering of fasted blood glucose and improved glucose tolerance upon a challenge, observed on a standard chow diet, prompted us to test the impact of enhanced metabolic stress on this model.



1E) Adiponectin levels from mice after 4 weeks of doxycycline containing chow diet (N=3 each group)

AiPKO mice are resistant to high fat diet-induced insulin resistance

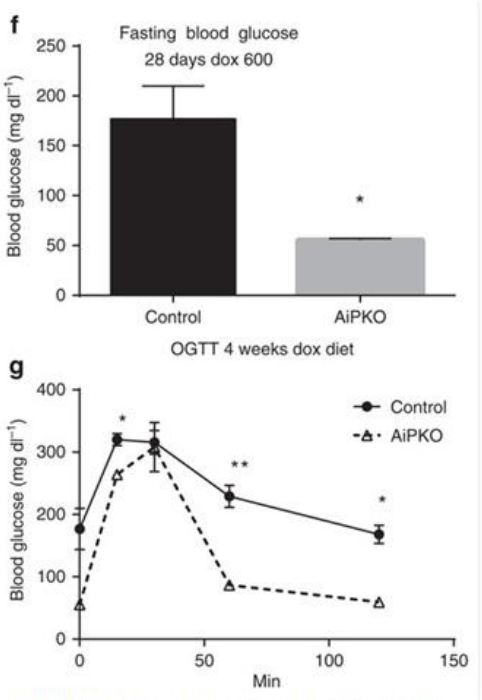
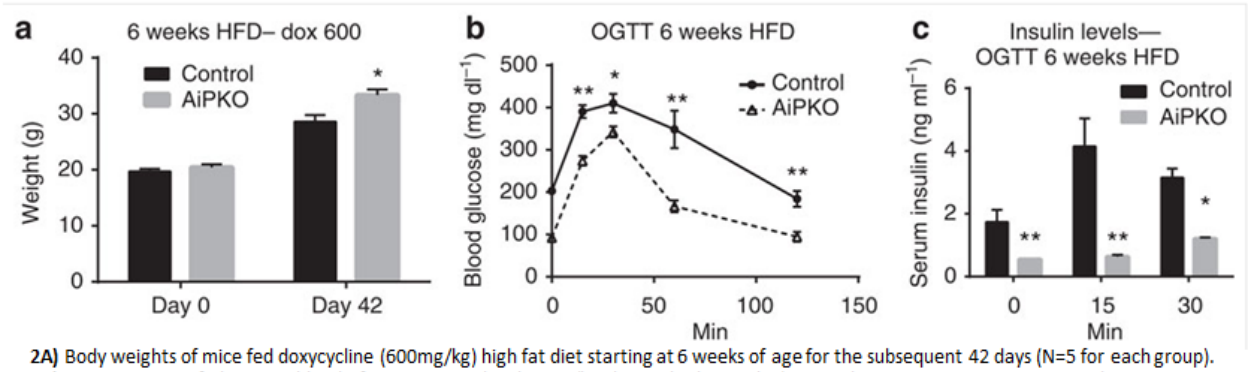


Fig1F) Blood glucose of mice fasted for 4 hours (N=4 each group). **Fig1G)** Oral glucose tolerance test of mice on doxycycline containing chow diet for 4 weeks

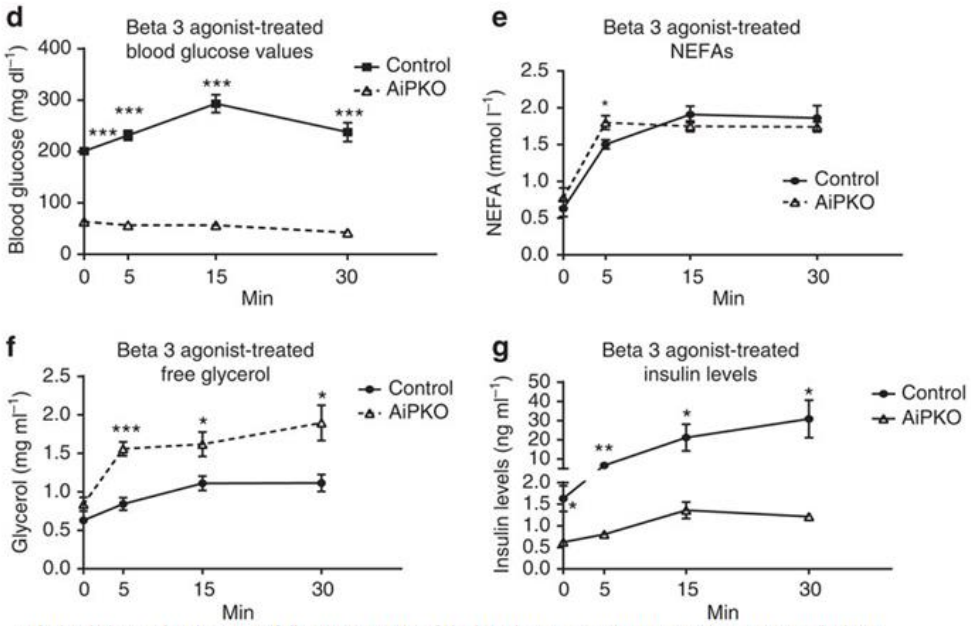
AiPKO mice, starting around 6-7 weeks of age, were switched to a high fat diet (HFD) containing 600mg/kg doxycycline. After 6 weeks of the HFD challenge, AiPKO mice had gained significantly more weight compared to controls (**Fig. 2A**). At that time, we subjected the mice to an oral glucose tolerance test. Mice were fasted for 4 hours and then gavaged with 2.5 g/kg glucose (**Fig. 2B**). The AiPKO mice demonstrated significantly improved glucose tolerance compared to controls despite the overall weight increase. We also assessed insulin levels over the course of the glucose challenge. AiPKO mice displayed much lower insulin levels compared to their wildtype littermates (**Fig. 2C**), consistent with an overall improvement in systemic insulin sensitivity.



2A) Body weights of mice fed doxycycline (600mg/kg) high fat diet starting at 6 weeks of age for the subsequent 42 days (N=5 for each group). **2B)** Concentration of glucose in blood of mice gavaged with 2.5 g/kg glucose body weight (n=5 each group, Average AUC; Control- 37827, AiPKO- 22877, P-value=.0011) **2C)** Serum Insulin concentrations of mice during oral glucose tolerance test shown in **B**

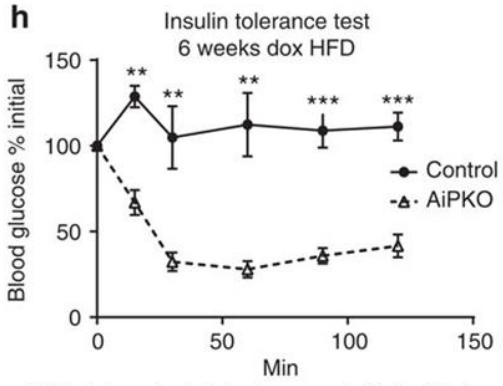
As the AiPKO mice displayed much lower insulin levels during the glucose challenge, we wished to determine whether this decreased insulin secretion was also observed in the setting of a pharmacologic challenge. Mice were subjected to a β3-adrenergic receptor agonist stimulation (with compound *CL316,243*), injected intraperitoneally following a 4 hour fast. This β3 adrenergic receptor stimulation leads to enhanced lipolysis, as well as a rapid and massive release of insulin (10). Over the course of the experiment, the AiPKO mice displayed reduced glucose levels, resulting in significant *hypo*glycemia over the entire course of the experiment (**Fig. 2D**). In contrast, the wildtype mice displayed *hyper*glycemia at all times. At every time point, except for the 5 minute mark, both wildtype and AiPKO mice overlapped with respect to

the increase in lipolysis as judged by significantly increased non-esterified fatty acids (NEFAs) in circulation; in contrast however, the AipKO mice demonstrated a disproportionately higher level of glycerol release (**Figs. 2E and 2F**). Interestingly, the NEFA levels in both genotypes were overlapping, even though the insulin levels in the AIPKO mice were dramatically lower compared to wildtype controls (**Fig. 2G**). In light of the fact that there are no β3 adrenergic receptors present on rodent β cells that could mediate the insulin release, these results further underscore our previous suggestion that insulin release from the β cell under these conditions is independent of NEFAs derived from adipocytes, consistent with the release of another secreted factor from adipocytes or a neuronally-mediated pathway mediating β3 adrenergic receptor stimulation in adipocytes to prompt insulin release from β-cells.



2D) Blood glucose levels in mice following injection of the b3 adrenergic agonist *CL316,243* at 0.5 mg/kg (n=4 control and 5 AiPKO). **2E)** NEFA **2F)** free glycerol and **2G)** insulin levels in the mice from **D)**.

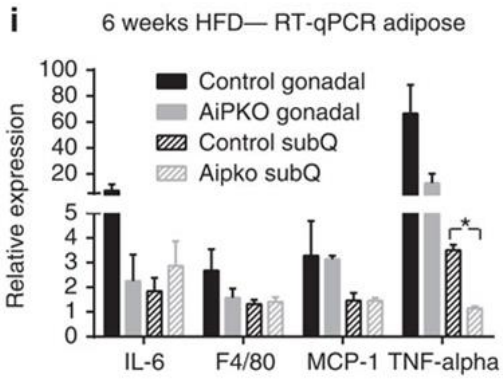
As these mice demonstrated dramatically lower insulin levels during both the OGTT as well as the β3-adrenergic stimulation, we wished to assess whole body insulin sensitivity after 6 weeks of HFD feeding. Mice were fasted for 4 hours and then injected with a submaximal dose of insulin (0.5 IU/Kg). Blood glucose was monitored at 15, 30, 60, 90 and 120 minutes. The AiPKO mice demonstrated a much



2H) Blood glucose levels displayed as a percent of fasting following injection with insulin (0.5 IU/kg)

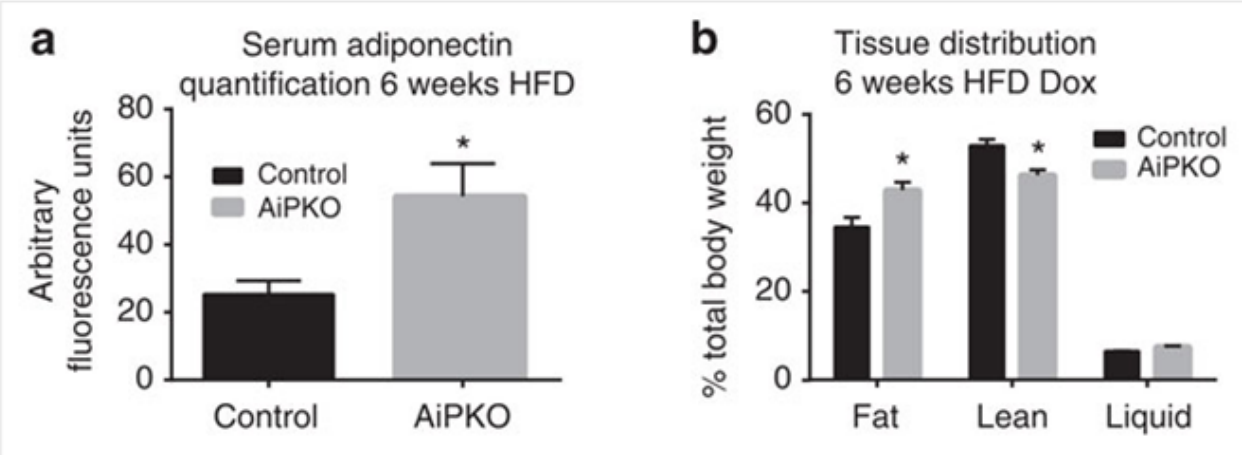
greater reduction in blood glucose compared to their wildtype littermates (**Fig. 2H**). This data suggests that these mice are significantly more insulin sensitive and that alterations of adipose tissue insulin sensitivity is sufficient to completely ameliorate the insulin resistance brought on by high fat diet feeding.

To determine any changes with respect to adipose tissue homeostasis at the cellular level, gonadal and subcutaneous adipose depots were isolated. qPCR analysis was performed on cDNA samples obtained from these fat pads in an effort to gauge alterations in the inflammatory tone, commonly elevated following HFD exposure. Among the genes tested, TNFα was significantly reduced in the subcutaneous adipose tissue of th e AiPKO mice compared to wildtype mice, and measurements in gonadal tissue showed similar trends (p=0.08) (**Fig. 2I**). This is a reflection of the overall improved health status of the fat pads in the AipKO mice.



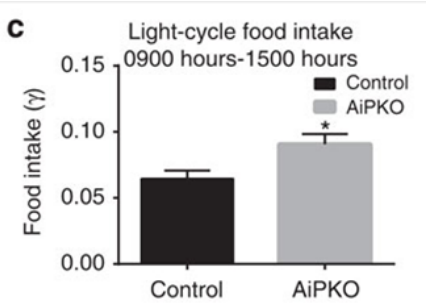
2I) Inflammatory gene expression in adipose tissue from mice fed HFD doxycycline containing diet for 6 weeks (N=3 each group).

Since the AiPKO mice demonstrated increased weight gain with improved glucose tolerance following the HFD challenge, we assessed whether this leads to any alterations in circulating adiponectin. Despite the widely-established inverse relationship of fat mass with adiponectin levels in circulation, and the generalized increased overall adiposity prompted by the lack of PTEN in the adipocyte, AiPKO mice placed on HFD containing doxycycline for 6 weeks demonstrated significantly higher serum adiponectin compared to controls (**Fig. 3A**). NMR analysis of these mice revealed an increase in total body adiposity with a concomitant relative decrease in lean body mass (**Fig. 3B**).



3A) Serum adiponectin levels of mice fed HFD doxycycline diet for 6 weeks, quantified using a Licor Western blot scanner (N=6 control and 4 AiPKO) **3B)** Tissue distribution determined by Bruker MQ10 NMR analyzer expressed as percent of total body weight (N=5 both groups).

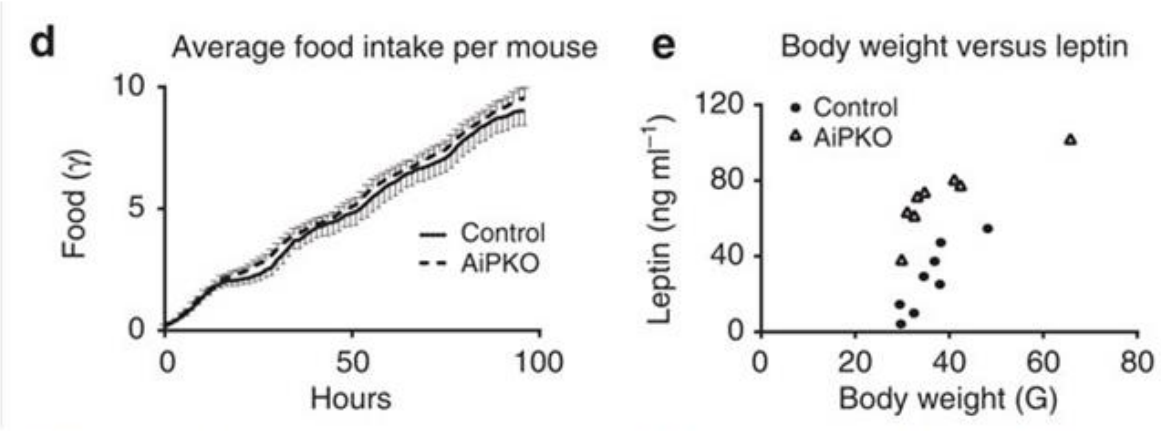
Metabolic Expenditure Analysis



3C) Average food intake per mouse per hour during the light cycle (sleep cycle) in a metabolic cage analysis. (N=4 control and 6 AiPKO).

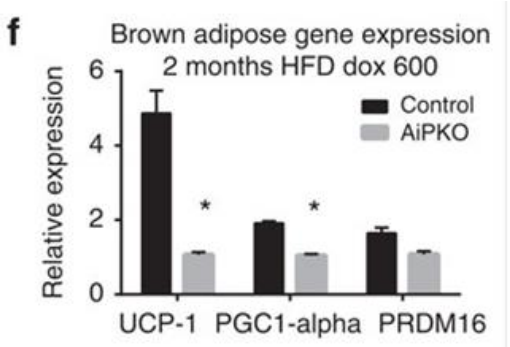
In an effort to identify the reasons responsible for the increased fat mass in the AiPKO mice, we subjected a separate cohort to analysis of metabolic chambers. 6 week-old AiPKO mice and their wildtype littermates were placed on HFD-doxycycline containing food. 16 days later, mice were placed into metabolic chambers for acclimatization and on day 20 following the initiation of HFD, data collection was initiated. AiPKO mice ate significantly more during the middle of the daytime sleep cycle (from 9am-3pm on a standard 6am-6pm light cycle) (**Fig. 3C**). However, of note is that the overall food intake integrated over the course of the entire 24 hour period was not changed. As the central hypothalamic axis is responsible for food intake, we sought to determine whether the AiPKO mice had alterations in the anorectic hormone leptin. Serum was collected from mice which had been fed HFD containing doxycycline (600mg/kg) for 8 weeks. This serum was analyzed by ELISA for leptin and demonstrated that the AiPKO mice had elevated leptin levels compared to the control mice when controlling for body weight (**Fig. 4E**). This was despite the lack of changes in overall food consumption during metabolic cage analysis (**Fig. 4D**) and did not correlate with leptin receptor expression in peripheral tissues (data not shown). This data suggests that although enhanced

insulin signaling in adipose tissue may increase leptin secretion, these mice still exhibit leptin resistance.



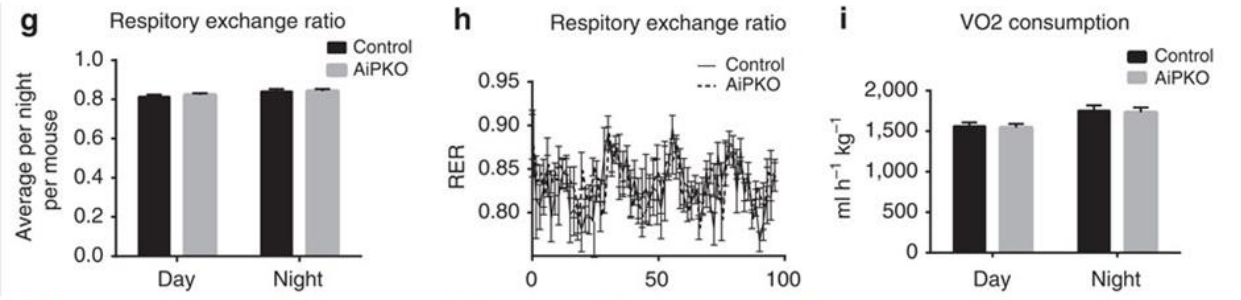
3D) Average total food intake per mouse over the 4 days of metabolic cage analysis. (N=4 control and 6 AiPKOs). **3E)** Circulating leptin levels versus body weight in the AiPKO and Control mice.

In an effort to identify other mechanisms of increased weight gain in the AiPKO mice, we looked at other metabolically active tissues. A primary mediator of energy expenditure is the activity of brown adipose tissue (BAT). This thermogenic adipose depot is capable of dissipating a great deal of energy and we determined whether our mice had any alterations in BAT as previous reports using the adiponectin-rtTA mouse demonstrated some activity in at least a subset of brown adipocytes (9). AiPKO and control mice were placed on a HFD containing doxycycline (600mg/kg) for 8 weeks. At this time, the mice were sacrificed and RNA was extracted and cDNA synthesized from the brown adipose tissue. RT-qPCR analysis of the brown adipose tissue revealed a significant down regulation of brown adipose associated genes including UCP-1, PGC1-α and PRDM16 in the AiPKO mice compared to controls (**Fig. 3F**). This data indicates a potential decrease in overall energy expenditure potentially explaining the increased weight gain observed. However, the AiPKO mice did not display any significant alterations in VO_2,



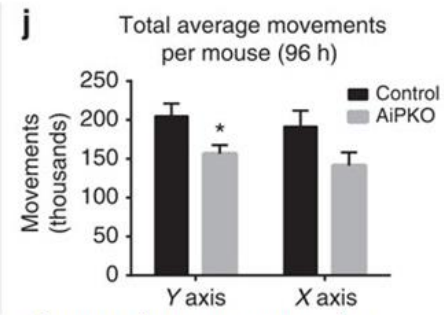
3F) Gene expression in brown adipose tissue following 6 weeks of doxycycline (600mg/kg) containing diet (N=3 control and 2 AiPKO)

VCO_2, RER and overall food consumption during the metabolic cage analysis (**1D-F**).



3G) Respiratory exchange ratios (RERs) for day and night cycles averaged per 12 hour period per mouse. (N=4 control and 6 AiPKOs). **3H)** Average respiratory exchange ratios for each group taken every 1.5 hours over the 4 days of metabolic cage analysis (N=4 control and 6 AiPKOs). **3I)** Volume of consumed oxygen for day and night periods, averaged per mouse per kilogram. (N=4 control and 6 AiPKOs).

Despite the lack of significant alterations in these metabolic parameters, the AiPKO mice moved significantly less than the wildtype littermates in the y-axis, and showed a trend toward reduced movement in the x-axis (p=0.08) (**Fig. 3D**). This data suggest that decreased movement and increased food consumption during resting hours are likely to account for the increased overall weight gain seen in AiPKO mice.

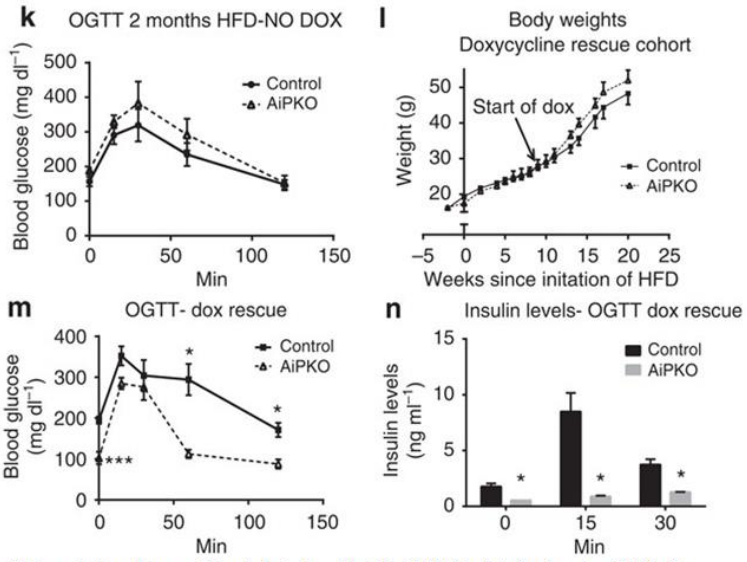


3J) Average total movements per mouse during metabolic cage analysis (96 hours). (N=4 control and 6 AiPKOs).

Loss of PTEN can restore insulin sensitivity after HFD exposure.

To determine if loss of PTEN from adipocytes could rescue a pre-existing diabetic phenotype induced by high fat diet feeding, AiPKO mice and their wildtype littermates were subjected to 8 weeks of HFD feeding starting at 6 weeks of age. At 8 weeks post initiation of HFD, an oral glucose tolerance test revealed no differences between the control mice and the AiPKOs (**Fig. 3K**). Mice were then switched to a HFD doxycycline containing diet and were monitored longitudinally. While the weights always trended towards a slight increase in the AiPKO mice after initiation of dox exposure, these differences never reached statistical significance (**Fig. 3I**).

At 4 weeks following the switch to doxycycline containing chow, mice were again subjected to an oral glucose tolerance test. The AiPKO mice demonstrated significant improvements compared to their control littermates (**Fig. 3m**). As before, insulin levels in these AiPKO mice were significantly lower than the controls (**Fig. 3n**). These observations indicate that the loss of PTEN in the adipocyte, i.e. a selective and inducible improvement in adipocytes *post* exposure to HFD, prompts a reversal of a pre-existing, established diabetic phenotype.

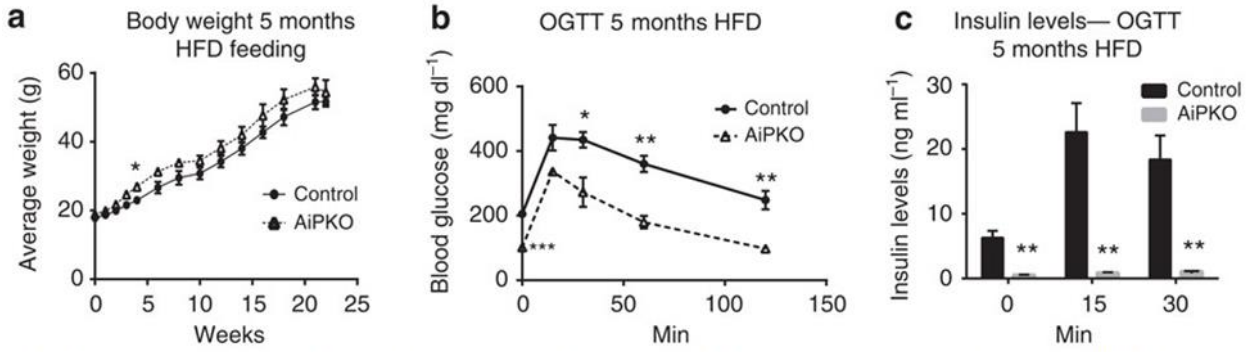


3K) Concentration of glucose in blood of mice from 3l) during OGTT (2.5 g/kg) after 8 weeks of HFD feeding without doxycycline (Average AUC; Control- 27725, AiPKO- 32702, P-value= 0.4246) 3m) Concentration of glucose in blood of mice from 3n) during OGTT (2.5 g/kg) after 8 weeks of HFD feeding without doxycycline followed by 4 weeks of HFD feeding containing Doxycycline (600 mg/kg)

AiPKO mice display improvements even after a long-term HFD insult

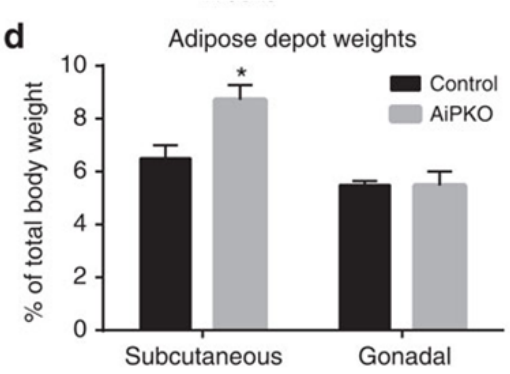
To determine whether the metabolic advantages conferred upon the AiPKO mice due to the absence of PTEN were long-lasting or whether some resistance to high PTEN starts to appear over time, mice were subjected to prolonged HFD feeding. Body weights of AiPKO mice were significantly elevated after 4 weeks and displayed a trend towards an increase in body weights compared to their control littermates over 24 weeks, though never reaching statistical significance thereafter (**Fig. 4A**). After 5 months of HFD feeding, AiPKO mice were subject to an oral glucose tolerance test (OGTT) (**Fig. 4B**). Even after this prolonged metabolic insult, the AiPKO mice handled a glucose bolus much more effectively. It is also apparent that the baseline fasting glucose levels (at the beginning of the OGTT) were much lower than in the control group. Furthermore, insulin levels during the OGTT were a fraction of the levels in the control mice, further illustrating a highly stable phenotype that maintains dramatic improvements in

metabolism even after a prolonged metabolic insult (**Fig. 4C**).

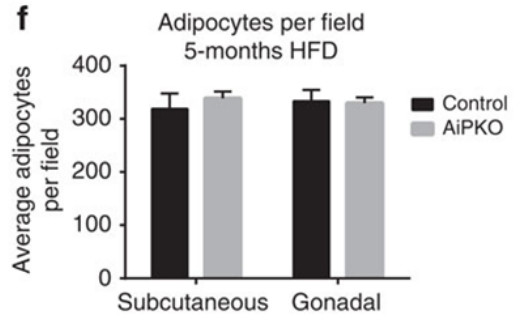


4A) Body weights of HFD doxycycline (600 mg/kg) fed mice for 23 weeks (N=4 each group). **4B)** Concentration of glucose in blood of mice gavaged with 2.5 g/kg body weight (n=4 each group). (Average AUC; Control- 41601, AiPKO- 22960, P-Value= 0.0007) **4C)** Insulin levels of mice during oral glucose tolerance test shown in **B**

Insulin was injected into these mice after a 4 hour fast. Fat pads were then harvested, and their weights assessed. AiPKO mice had considerably larger subcutaneous fat depots, while the gonadal tissue was unaffected (**Fig. 4D**). As adipose tissue can expand via hypertrophy or hyperplasia, we analyzed sections of both gonadal and subcutaneous adipose sections for cell number (**Fig. 4F**). This analysis revealed similar numbers of cells per field in the sections, regardless of genotype. We conclude therefore that the significant expansion of the subcutaneous adipose depot in the AiPKO mice was due predominantly to hyperplasia, a distinctive feature of healthy adipose expansion.



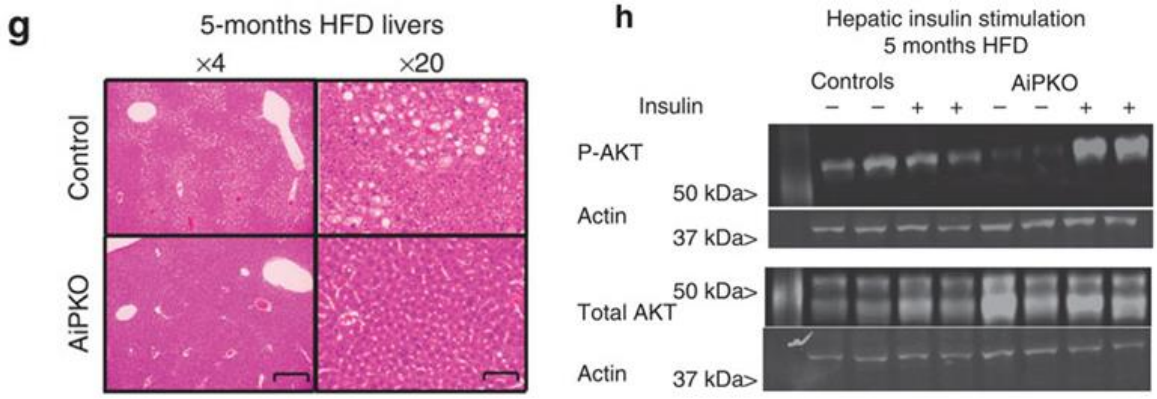
4D) Adipose depot weights at the time of sacrifice for each cohort (n=4 both groups).



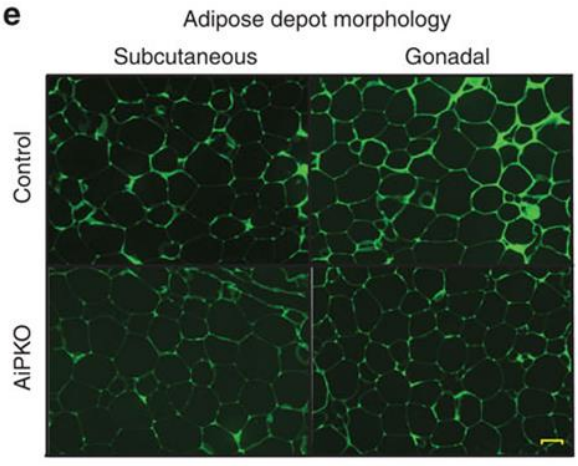
4f) Quantification of the number of adipocytes observed per field at 4x magnification from the subcutaneous and gonadal adipose depots of both control and AiPKO mice (N=2 mice from each group (Control and AiPKO) and 3 fields of view for each tissue section, total 6 fields per group)

In parallel to the experiments above, we determined whether the loss of PTEN in adipose tissue was protective for the development of fatty liver after 5 months of HFD feeding. Histological examination of the liver demonstrated significant fat accumulation in the control mice. This stood

in stark contrast to the situation in the AiPKO mice, which lacked fat accumulation in their livers after 5 months of high fat diet feeding (**Fig. 4g**). To determine whether this lack of lipid accumulation had functional implications, the livers from the insulin-stimulated mice were also analyzed for phospho-AKT levels. AiPKO mice showed significantly greater AKT phosphorylation compared to their wildtype littermate controls (**Fig. 4h**). Of note is the fact that total AKT levels also increase quite substantially in the AiPKO mice. This demonstrates the significant protective effects on hepatic insulin sensitivity upon selective improvements in insulin-sensitivity in adipocytes.



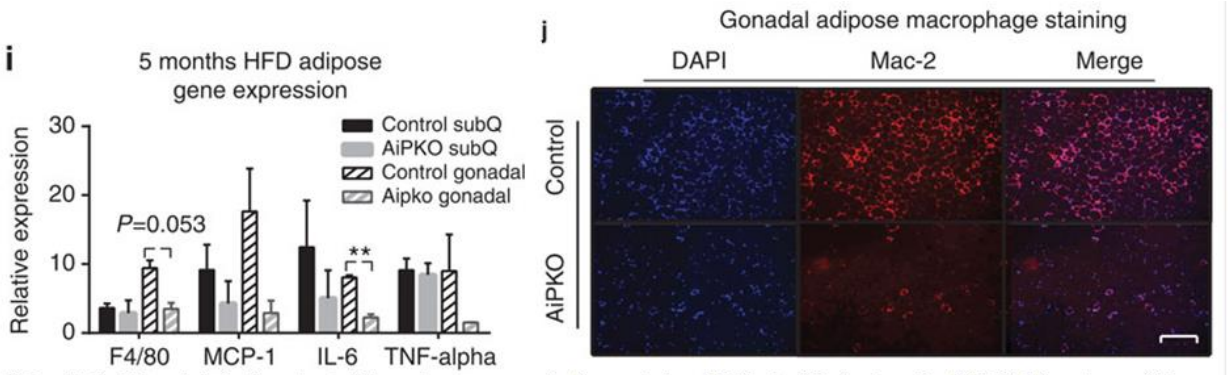
4g) Liver histology (H&E stain) of control and AiPKO mice after 5 months of HFD Doxycycline feeding. **4h)** Western blot of liver protein extracts from mice treated with PBS or insulin for 15 minutes prior to sacrifice (N=4 for each group, 2 receiving insulin and 2 controls)



4e) Auto fluorescent image (excitation at 480nm) of adipocytes from AiPKO and control mice

To determine whether the long-term high fat diet feeding had caused lasting alterations at the cellular level in the adipose tissue, gross structural examination under auto-fluorescent conditions revealed no change in adipocyte size or number (**4E)**. To further confirm any alterations to the adipose tissue the fat pads of these mice were subjected to RT-qPCR and immunofluorescence analysis. RT-qPCR data shows decreased F4/80 expression and significantly less IL-6 production in the gonadal fat depots of AiPKO mice, without significant

changes of these immune markers in the subcutaneous depot (**Fig. 4G**). In contrast to the short-term HFD feeding, the long-term HFD feeding did not result in statistically significant changes in TNFα expression. To further confirm the increase in macrophage accumulation, immuno-fluorescent staining for Mac2 in the gonadal fat pad was performed. This analysis demonstrated highly elevated macrophage infiltration in the control mice versus the AiPKO, confirming at the protein level the RT-qPCR analysis (**Fig. 4H**).



G) Quantitative PCR analysis of adipose depots inflammatory gene expression from controls and AiPKO mice following 5 months of HFD (N=3 for each group) **H)** Gonadal adipose depot staining for macrophage infiltration after 5 months of HFD feeding using an anti-Mac2 antibody with a DAPI counterstain.(Experiment was performed on N=3 mice for each group).

Discussion

Only one previous manipulation of the insulin receptor cascade leading to an *increase* in insulin signaling in the adipocyte has been described (11), and none of the critical constituents have been inducibly eliminated selectively from the mature adipocyte only. In the report by Kurlawalla-Martinez and colleagues, the authors used a constitutive aP2-cre line to eliminate PTEN from adipocytes. Their results differed substantially from ours, and these differences likely stem from two major sources: aP2-driven Cre expression may extend to cell types in other tissues under some circumstances, though this can be controlled for (12). More importantly, aP2-driven transgene expression can eliminate target genes in early pre-adipocyte progenitors, thereby leading to a developmental phenotype that masks the role of the target protein in the mature adipocyte. The latter of these effects presumably resulted in compensatory developmental adaptations. As a result, these authors failed to see changes in body weight or fasting blood glucose levels, both of which are highly apparent upon elimination of PTEN exclusively from the mature adipocyte, and lead to an underestimation of the relevance of PTEN action in the adipocyte.

To put our observations into additional context, another manuscript on the topic was previously published by Komazawa and colleagues (13). The phenotype described for their adipocyte-specific PTEN null model (employing a constitutive adiponectin promoter- driven Cre) was fundamentally different from what we describe here, and more importantly, these observation were not reproducible, and the paper was subsequently retracted (14).

The use of inducible transgenic models therefore allows for the evaluation of gene function in the adult mouse and enables a more accurate depiction of the physiological impact of a given protein as compared to congenital loss-of-function approaches that affect the development of the tissue. To achieve this in the context of adipose tissue insulin signaling, we used an

adiponectin promoter driven rtTA cassette coupled with a TRE-CRE transgenic mouse to inducibly eliminate a floxed PTEN locus from mature adipocytes. Upon confirming that our inducible model effectively and specifically leads to loss of function of PTEN in adipocytes, we assessed the impact of this alteration on mature adipocytes locally at the cell autonomous level as well as its impact on systemic metabolism. Overall, the loss of PTEN in the mature adipocyte leads to increased insulin sensitivity in multiple peripheral tissues, and a greatly improved metabolic phenotype upon acute and chronic exposure to high fat diets. This demonstrates that selective improvements in insulin sensitivity at the level of the adipocyte are sufficient to improve whole body glucose homeostasis.

In clinical studies as well as rodent and non-human primate studies, the levels of the circulating protein adiponectin have been tightly correlated with insulin sensitivity (6). Here, we have the surprising finding that animals with highly insulin sensitive adipose tissue lack a corresponding increase in circulating adiponectin under basal conditions. As the adipose tissue in the AiPKO mice demonstrates increased insulin sensitivity in both arms of the insulin response pathway following HFD challenge, not only the PI3-AKT branch, we argue that insulin sensitivity in adipose tissue *per se* is not sufficient to mediate the regulation of circulating adiponectin (**Fig. 1D**). This data is supported by clinical studies demonstrating that subjects producing endogenous anti-insulin receptor antibodies display massively elevated circulating adiponectin despite a very high level of insulin resistance (7). Many questions remain as to how the release of adiponectin is regulated by local insulin sensitivity, but a number of studies hint at mitochondrial metabolism playing a major role in determining how much adiponectin is produced and released from the adipocyte (15).

Upon challenging our mice with a HFD, a condition which caused the AiPKO mice to gain significantly more weight, we saw increased circulating adiponectin relative to the control mice. With their greater percent body fat and increased insulin sensitivity these mice do not display

the drastic decrease in circulating adiponectin commonly seen in insulin resistant states. This observation is highly reminiscent of the clinical setting in which patients exposed to the antidiabetic drug thiazolidinediones have significantly elevated adiponectin levels despite increase fat mass (16). An alternative explanation is that rather than displaying increased adiponectin production on HFD, the AiPKO mice may simply be resistant to the decrease in production commonly seen in wildtype mice. Therefore, circulating adiponectin may be more reliably used as a marker of insulin resistance, rather than obesity.

Selective activation of β3 adrenergic receptors with the agonist *CL316,243* is known to acutely induce adipose tissue lipolysis, increase circulating free fatty acids and insulin. An increase in circulating FFAs can cause a direct activation of GPR40 on β cells, inducing granule exocytosis and increasing circulating insulin levels in the mouse (10). We injected a low dose of the selective β3 agonist to the AiPKO mice and the controls. Although the circulating glycerol levels are significantly greater in AiPKO mice following stimulation, the NEFA levels are only mildly elevated at 5 minutes and equal at all subsequent time points. Therefore, if insulin release is triggered by NEFAs, equal amounts of insulin are expected to be released from the β cells. However, this is not the case. Despite similar levels of circulating NEFAs in AiPKOs following β3 stimulation, their insulin levels are dramatically lower than what is seen in wildtype controls. This brings to bear strong disconnect in the current model of β3 agonist-induced insulin secretion. It remains unclear at this time what the mechanism of β3 agonist-induced insulin secretion is. There are no β3 adrenergic receptors in murine β cells, so this has to be an effect mediated by receptor activation at the level of the white adipocyte (17). Yet, the results shown here rule out a direct effect of NEFAs.

Chronic high fat diet feeding is highly associated with inflammatory adipose tissue (18). This inflammatory response is most notably dominated by increased macrophage infiltration and

concomitant increases in local TNFα and IL-6 levels (19). Previous reports have demonstrated that systemic inhibition of this inflammatory response through genetic through genetic means can improve the metabolic homeostasis of animals (19). The AiPKO model demonstrates that upon maintaining adipose insulin sensitivity during HFD feeding, there is a dramatic reduction of the inflammatory response commonly observed under these conditions. Upon a short term HFD challenge (6 weeks), we see a significant reduction in TNFα secretion in the adipose depot, specifically in the subcutaneous depot. Following long-term high fat diet feeding (5 months), we see a significant reduction in the maladaptive inflammatory response in the gonadal depot as indicated by decreased overall expression of the macrophage marker F4/80, decreased IL-6 production, and decreased crown-like structure staining. These observations indicate that the maladaptive inflammatory response to high fat diet feeding is dictated, directly or indirectly, by adipose tissue insulin sensitivity. The improved local insulin sensitivity is potently cytoprotective for adipocytes, leading to reduced necrotic adipocyte cell death. This in turn leads to the reduced infiltration of macrophages infiltration and the long-term improvements in insulin sensitivity. Further, decreases in adipose tissue inflammation are known to dictate inflammatory responses in other metabolically active tissues (20). Therefore, these findings represent a potential mechanism by which maintenance of insulin signaling in adipose tissue may reduce inflammation at distant sites.

Not unexpectedly, maintaining healthy adipose tissue function in AiPKO mice leads to potent liver-protective effects during high fat diet exposure. Adiponectin with its potent hepatoprotective actions is likely to be at least partially responsible for this phenomenon (5). Following 5 months of HFD feeding, the AiPKO livers display significantly less hepatic steatosis compared to controls. Furthermore, the livers are exquisitely sensitive to insulin stimulation as judged by increased phosphoAKT staining following IP insulin injection. This model represents an additional case of adipose tissue as a primary driver of whole body insulin sensitivity and

metabolism. Many examples from our own work as well as work from others have illustrated this point, such as the observations by Kahn and colleagues who knocked out the glucose transporter Glut4 specifically from adipocytes, leading to profound systemic disturbances (4, 21).

The model presented here is also in support of the concept that expansion of adipose tissue mass (i.e. obesity) is in fact a defensive mechanism against caloric overload. We see here that protection from diabetes during caloric overload leads to expansion of the subcutaneous depot, commonly referred to as “healthy” fat expansion. As previous studies in our laboratory have shown, expansion of adipose can have potent protective effects from the metabolic derangements seen during HFD induced insulin resistance (13, 18). Interestingly, in the AiPKO mice, already healthy insulin sensitive adipose expands greatly in the subcutaneous depot as opposed to the gonadal. This is another example of the potent anti-lipotoxic effects that subcutaneous depots can confer upon the system upon “healthy” expansion.

The neutralization of excess calories by effectively storing them in adipose tissue is a central aspect of the anti-lipotoxic actions of adipose tissue expansion. Maintaining a high level of insulin sensitivity in the adipocyte is a crucial ingredient in the complex set of reactions that lead to the “healthy” expansion of adipose tissue that mediates the potent insulin-sensitizing effects of the process. Keeping the insulin-sensitivity at a high level keeps adipose tissue properly vascularized, maintains an adequate number of adipocytes and keeps the level of extracellular fibrosis at bay. The selective loss of PTEN at the level of the mature adipocyte is sufficient for this process.

Materials and methods

Animals

Animal care and experimental protocols were approved by the Institutional animal care and use committee of the University of Texas Southwestern medical center. Adiponectin-RTTA mice were produced as previously described (9). Tre-Cre and PTEN floxed mice were purchased from Jackson laboratories. All experiments were done using male mice with littermate controls that were adiponectinP-rtTA or TRE-cre negative. Mice were fed a standard chow diet (number 5058, LabDiet), a Dox-chow diet (600 mg kg^{-1} Dox; Bio-Serv), a 60% HFD (D12492, Research Diets Inc.) or a Dox-HFD (600 mg kg^{-1} Dox; Bio-Serv). Following weaning mice were randomly placed into cages for the remainder of their life before genotyping was performed. No other randomization was used while conducting experiments.

Statistical analysis

All animal studies were designed to minimize and control for confounding variables such as mouse strain, gender, age, time of day, fasted/fed state, diet and light cycle. Based on previous studies utilizing animal models for metabolic research, we can utilize as few as 6 animals per treatment group to achieve statistical power to detect significant differences when measuring RNA, protein and blood metabolites. With the use of inducible mouse models, group sizes were increased to 10 to account for any potential variation imposed by the use of ingested gene induction agents. Metabolic cage studies were performed by a technician who was blinded as to the genotype of the mice. For all other animal work, researchers were not blinded as to genotype.

UT Southwestern Medical Center has a site license for the use of GraphPad Prism software (version 6, GraphPad, Inc. San Diego CA). All data is depicted as mean plus and minus the SEM. All experiments presented have only two groups and unpaired students T-test was utilized

for statistical significance. All data compares Control mice to AiPKO mice under identical conditions. For all points * represents P-value<0.05, ** represents P-value<0.01, *** represents P-value<0.001 .

Metabolic testing

Oral glucose tolerance tests were performed as previously described (15). Briefly mice were fasted for 4 hours before being a gastric gavage of glucose at 2.5 g/kg body weight. Glucose concentrations were measured using an oxidase-peroxidase assay (Sigma). Insulin and leptin levels were measured using commercial ELISAs from Millipore. Systemic insulin sensitivity was tested by performing an insulin tolerance test. Mice were fasted for 4 hours, and then injected with 0.5 IU/kg body weight of Humalin-R (Eli Lilly). Blood from the tail was measured for glucose content using Contour glucometer strips (Bayer) at 0, 15, 30, 60, 90 and 120 minutes. β3 adrenergic receptor tests were performed as previously described (15). Briefly, mice were fasted for 4 hours, at which time *CL316,243* was administered intraperitoneally at 0.5 mg/kg body weight. Blood was collected at 0, 5, 15 and 30 minutes. Glycerol and NEFA measurements were determined using free glycerol reagent (Sigma) and free fatty acid quantification kits (Wako Diagnostics-NEFA-HR2) respectively.

Quantitative RT-qPCR

RNA was isolated from tissues frozen on liquid nitrogen by homogenization in Trizol Reagent (Invitrogen) and RNA extraction using a RNAeasy RNA extraction kit (Qiagen). RNA was quantified using a Nanodrop Instrument. 800ng of RNA was used to transcribe cDNA using an Iscript kit (Invitrogen). RT-qPCR primers are listed in Supplementary Table 1. The mRNA levels were calculated using the comparative threshold cycle (Ct) method.

Western blotting

Frozen tissue was homogenized in TNET buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 with phosphatase inhibitors and protease inhibitors as described in (22) (Sigma). For adipose tissue samples, tissue was homogenized first in the absence of Triton X-100, the fat layer extracted, and Triton X-100 was then added to a final concentration of 1%. Proteins were resolved on 11% Tris-Glycine gels and then transferred to nitrocellulose membranes (Bio-Rad). Adiponectin was detected with a rabbit polyclonal antiserum (23). pAkt (Ser473, antibody #4060) and total Akt (antibody #2920) (Cell Signaling Technology) were used (1:1,000) for insulin signaling studies. Primary antibodies were detected using a secondary IgG labeled with infrared dyes emitting at 700 nm (Li-Cor Bioscience #926-32220) or 800 nm (Li-Cor Bioscience #926-32211) (both at 1:5,000 dilutions) and then visualized on a Li-Cor Odyssey infrared scanner (Li-Cor Bioscience). The scanned data were analyzed using Odyssey Version 2.1 software (Li-Cor Bioscience).

Histology and immunofluorescence

Fat pads and livers were excised and fixed overnight in 10% PBS buffered formalin and were thereafter stored in 50% ethanol. Tissues were sectioned (5um) rehydrated and stained using H+E, or with primary antibodies to PTEN (Abcam 32199) or Mac-2 (CL8942AP, CEDARLANE Laboratories USA Inc.). Slides were mounted using Prolong Gold Antifade reagent with DAPI (Life Technologies).

NMR and Metabolic Cages

Metabolic measurements were obtained continuously using TSE metabolic chambers (TSE Labmaster System, Germany) in an open circuit indirect calorimetry system. Data was normalized to lean body mass as determined using a Bruker MQ10 NMR analyzer.

Adipocyte isolation

Adipocytes were isolated for confirmation of gene loss and determination of cell autonomous insulin sensitivity using a collagenase digestion similar to SVF isolation procedures (24). Briefly, freshly isolated adipose tissue was minced and subjected to 1 hour digestion at 37ºC in a PBS-collagenase buffer pH 7.4 (collagenase sigma (10mg/ml). This digest was centrifuged at 600xg for 5 minutes and the floating cells removed for RNA extraction.

References

1. Nawrocki AR, Scherer PE. The delicate balance between fat and muscle: adipokines in metabolic disease and musculoskeletal inflammation. Current opinion in pharmacology. 2004;4(3):281-9. Epub 2004/05/14. doi: 10.1016/j.coph.2004.03.003. PubMed PMID: 15140421.
2. Bluher M, Michael MD, Peroni OD, Ueki K, Carter N, Kahn BB, et al. Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. Developmental cell. 2002;3(1):25-38. Epub 2002/07/12. PubMed PMID: 12110165.
3. Liu M, Bai J, He S, Villarreal R, Hu D, Zhang C, et al. Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1. Cell metabolism. 2014;19(6):967-80. Epub 2014/04/22. doi: 10.1016/j.cmet.2014.03.018. PubMed PMID: 24746805; PubMed Central PMCID: PMC4064112.
4. Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, et al. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature. 2001;409(6821):729-33. Epub 2001/02/24. doi: 10.1038/35055575. PubMed PMID: 11217863.
5. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nature medicine. 2001;7(8):947-53. Epub 2001/08/02. doi: 10.1038/90992. PubMed PMID: 11479628.
6. Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G. Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. Diabetes. 2003;52(7):1779-85. Epub 2003/06/28. PubMed PMID: 12829646.
7. Semple RK, Halberg NH, Burling K, Soos MA, Schraw T, Luan J, et al. Paradoxical elevation of high-molecular weight adiponectin in acquired extreme insulin resistance due to insulin receptor antibodies. Diabetes. 2007;56(6):1712-7. Epub 2007/02/28. doi: 10.2337/db06-1665. PubMed PMID: 17325257; PubMed Central PMCID: PMC2253187.
8. Peyrou M, Bourgoin L, Poher AL, Altirriba J, Maeder C, Caillon A, et al. Hepatic PTEN deficiency improves muscle insulin sensitivity and decreases adiposity in mice. Journal of hepatology. 2014. Epub 2014/09/23. doi: 10.1016/j.jhep.2014.09.012. PubMed PMID: 25234947.
9. Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nature medicine. 2013;19(10):1338-44. Epub 2013/09/03. doi: 10.1038/nm.3324. PubMed PMID: 23995282.
10. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, et al. Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature. 2003;422(6928):173-6. Epub 2003/03/12. doi: 10.1038/nature01478. PubMed PMID: 12629551.
11. Kurlawalla-Martinez C, Stiles B, Wang Y, Devaskar SU, Kahn BB, Wu H. Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. Molecular and cellular biology. 2005;25(6):2498-510. Epub 2005/03/04. doi: 10.1128/MCB.25.6.2498-2510.2005. PubMed PMID: 15743841; PubMed Central PMCID: PMC1061603.
12. Martens K, Bottelbergs A, Baes M. Ectopic recombination in the central and peripheral nervous system by aP2/FABP4-Cre mice: implications for metabolism research. FEBS letters. 2010;584(5):1054-8. Epub 2010/02/09. doi: 10.1016/j.febslet.2010.01.061. PubMed PMID: 20138876.
13. Komazawa N, Matsuda M, Kondoh G, Mizunoya W, Iwaki M, Takagi T, et al. Enhanced insulin sensitivity, energy expenditure and thermogenesis in adipose-specific Pten

suppression in mice. Nature medicine. 2004;10(11):1208-15. Epub 2004/10/19. doi: 10.1038/nm1117. PubMed PMID: 15489860.
14. Komazawa N, Matsuda M, Kondoh G, Mizunoya W, Iwaki M, Takagi T, et al. Retraction: Enhanced insulin sensitivity, energy expenditure and thermogenesis in adipose-specific Pten suppression in mice. Nature medicine. 2005;11(6):690. Epub 2005/06/07. doi: 10.1038/nm0605-691a. PubMed PMID: 15937475.
15. Kusminski CM, Holland WL, Sun K, Park J, Spurgin SB, Lin Y, et al. MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity. Nature medicine. 2012;18(10):1539-49. Epub 2012/09/11. doi: 10.1038/nm.2899. PubMed PMID: 22961109; PubMed Central PMCID: PMC3745511.
16. Combs TP, Wagner JA, Berger J, Doebber T, Wang WJ, Zhang BB, et al. Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. Endocrinology. 2002;143(3):998-1007. Epub 2002/02/28. doi: 10.1210/endo.143.3.8662. PubMed PMID: 11861525.
17. Grujic D, Susulic VS, Harper ME, Himms-Hagen J, Cunningham BA, Corkey BE, et al. Beta3-adrenergic receptors on white and brown adipocytes mediate beta3-selective agonist-induced effects on energy expenditure, insulin secretion, and food intake. A study using transgenic and gene knockout mice. The Journal of biological chemistry. 1997;272(28):17686-93. Epub 1997/07/11. PubMed PMID: 9211919.
18. Chalkiadaki A, Guarente L. High-fat diet triggers inflammation-induced cleavage of SIRT1 in adipose tissue to promote metabolic dysfunction. Cell metabolism. 2012;16(2):180-8. Epub 2012/08/14. doi: 10.1016/j.cmet.2012.07.003. PubMed PMID: 22883230; PubMed Central PMCID: PMC3539750.
19. Kawanishi N, Yano H, Yokogawa Y, Suzuki K. Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice. Exercise immunology review. 2010;16:105-18. Epub 2010/09/16. PubMed PMID: 20839495.
20. Seijkens T, Kusters P, Chatzigeorgiou A, Chavakis T, Lutgens E. Immune cell crosstalk in obesity: a key role for costimulation? Diabetes. 2014;63(12):3982-91. Epub 2014/11/22. doi: 10.2337/db14-0272. PubMed PMID: 25414012.
21. Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, et al. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. The Journal of clinical investigation. 2007;117(9):2621-37. Epub 2007/08/25. doi: 10.1172/JCI31021. PubMed PMID: 17717599; PubMed Central PMCID: PMC1950456.
22. Scherer PE, Lisanti MP, Baldini G, Sargiacomo M, Mastick CC, Lodish HF. Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. The Journal of cell biology. 1994;127(5):1233-43. Epub 1994/12/01. PubMed PMID: 7962086; PubMed Central PMCID: PMC2120260.
23. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. The Journal of biological chemistry. 1995;270(45):26746-9. Epub 1995/11/10. PubMed PMID: 7592907.
24. Gupta RK, Mepani RJ, Kleiner S, Lo JC, Khandekar MJ, Cohen P, et al. Zfp423 expression identifies committed preadipocytes and localizes to adipose endothelial and perivascular cells. Cell metabolism. 2012;15(2):230-9. Epub 2012/02/14. doi: 10.1016/j.cmet.2012.01.010. PubMed PMID: 22326224; PubMed Central PMCID: PMC3366493.

Chapter 3

Brown and Beige Adipose

Physiologic importance of brown adipocytes

Brown adipose tissue (BAT) is one of the most evolutionarily advanced organs in mammals and is one that is commonly overlooked as being essential for our success as a species. The ability for newborns and infant mammals to maintain body temperature has allowed the spread of mammals to many corners of the globe, commonly considered uninhabitable by larger species. This highly thermogenic tissue is capable of burning lipids and glucose in an effort to maintain core body temperature in response to central signals emitted from the hypothalamus. The role of BAT in thermogenesis has been appreciated for many years. More recently, its potential as a regulator of obesity and diabetes has caused a surge in the attention given to this previously underappreciated organ; particularly in the last 20 years beige adipocytes have garnered a lot of attention. (1), These “brown-like” adipocytes identified in the subcutaneous depot of the mouse share many similarities with brown adipocytes, yet are inducible with cold exposure in adult animals (2). Their ability to be induced and to burn fuel substrates such as glucose and lipids providing a potential for therapeutic benefit in diabetes have caused an enormous surge in the interest in these cells.

Brown versus beige Lineage

In adult humans, brown adipose can be identified by PET imaging in the upper neck and para-vertebral regions, where as in human infants and similarly in mice, the majority of brown adipose exists in the back of the animal near the shoulder blades in the interscapular region. These locations provide unique sites for thermogenesis and are well situated to aid in warming up a cold animal. In the mouse, brown adipocytes emerge from a My5-positive lineage, a lineage previously thought to be restricted to skeletal muscle (3). Moreover, these cells express a very distinct lineage marker, PRDM16, which is both

sufficient and necessary for their development. Loss of PRDM16 from the muscle cells forces them toward a myoblastic fate, while ectopic expression of PRDM16 in muscle cells leads to brown adipogenesis. PRDM16 is required not only for the formation of brown adipose tissue, but also for its maintenance; the loss of PRDM16 from the My5 lineage decreases EHMT1 recruitment and its function as a suppresser of the white adipogenic program (4).

Interestingly, both white adipocytes and their beige relatives do not originate from this distinct My5 lineage. Beige adipocytes have been shown to be exclusively targeted using the PGC1-α Cre mouse, though this is still debated in the field (5). Further, their precursors can be selected for using FACS analysis by isolating the populations of Lin-, CD29+, Sca1+, CD24+ cells (6). Beige adipocytes have clearly been demonstrated to emerge from these PGC1-α derived cells that are cd34+, sca1+ (7). β-adrenergic receptor stimulation drives the fate of these cells down this pathway; and although the experiments have not been done to date, it appears to be absolutely required for their development *in vivo*.

Developmental origins of brown and beige adipose

Brown adipocytes have been shown to arise from a developmental precursor that is both My5 and Pax 7 positive in the mouse (3, 8). The Myf5 lineage is most commonly associated with skeletal muscle, and brown adipocytes have been shown to have gene signatures that are similar to muscle (9). Although brown adipocytes have a different developmental precursor, they do share many similarities with white adipocytes including their expression of adiponectin and the presence of lipid droplet proteins including the Perilipin genes (2, 10). As brown adipose tissue is arranged neonatally and can expand in adult humans, much more interest has been generated toward understanding the inducible beige adipocytes.

Beige adipocytes emerge following cold stimulation, or pharmacologic treatment, almost exclusively restricted to the subcutaneous adipose depot. Similar to white and distinct from brown, beige adipocytes do not emerge from My5+ precursors *in vivo*. As recently demonstrated by our group, the

exposure of a mouse to 6º C causes the emergence of a distinct beige adipocyte population not derived from preexisting white adipocytes (2). This data clearly indicates *de novo* beige adipogenesis rather than direct trans-differentiation from white to beige adipocytes. Several groups have worked independently using different approaches in an effort to identify the source of these precursors.

Using primary clonal expansion and limiting dilution, Wu et al derived dozens of cell lines from the subcutaneous adipose depot of mice (11). They then identified multiple types of adipocytes that could be derived from these cell lines, and specifically found those which did or did not respond to cAMP increases by upregulating their beige/brown adipogenic gene profile. In these studies, they identified a panel of genes which are distinct to beige cells and those that are distinct to brown adipocytes. This work led to the identification of 2 cell surface markers which are unique for beige preadipocytes, CD137 and TMEM26.

In other efforts to identify beige precursors, Wang et al found that EBF2 was dramatically enriched in precursors of both brown and beige adipose tissue (12). They demonstrated that Myf5-cre labeled precursors were PDGFR-α +, and then upon isolating these cells, EBF2 was highly enriched in this population. Using an EBF2-GFP mouse, they then demonstrated that cells labeled in the subcutaneous white adipose tissue of these mice were beige adipocyte precursors. Interestingly, they showed that EBF2 regulates a majority of genes in brown preadipocytes. Taken together, these observations support the idea that we can now isolate populations of cells from the white adipose of mice (CD137+, PDGFRα+, EBF2+) that are specifically beige precursors.

Therapeutic potential of brown/beige adipose tissue in diabetes

As body weights and BMIs have trended up over the last 40 years in the general population along with the increase in diabetes, the field has looked at the ability of brown, and more importantly, beige adipose tissue to combat these problems. Excess energy substrates in our diets lead to an increased demand of insulin to deal with and store these molecules. As insulin resistance develops, the system is less capable of handling this metabolic role. Therefore, it is believed that the ability to dissipate the energy stored in adipose tissue and other sites in the form of ectopic lipid deposition (such as in the obese liver), will be associated with an improvement in overall metabolic homeostasis. This idea gained support when the Kozak group first demonstrated that overexpression of UCP1 in white adipose (via the aP2 promoter) was able to reduce obesity in rodents (13). These findings demonstrated the first potential therapeutic role for UCP1 expression.

The initial observations as to whether UCP1 mediated thermogenesis in brown adipose tissue had effects on body weight and energy expenditure came from the first reports of UCP1 knockout mice. Mice deficient for the UCP1 gene, and therefore in principle, incapable of thermogenic uncoupled mitochondrial respiration, were found to be cold intolerant, but not obese (14). These authors demonstrated that increased oxygen consumption following β adrenergic stimulation was deficient in these mice. However, they could not demonstrate hyperphagia or obesity. Interestingly, they suggested that UCP2 may compensate in the brown adipose of this knockout; however this seems doubtful as this would likely have masked the differences in β3-stimulated oxygen consumption.

Several years later, Barbra Cannon's group found that UCP1 knockout mice become obese when housed at thermo-neutrality as opposed to room temperature (15). This in itself is a very interesting finding, as UCP1 activity in brown and beige adipose should be entirely absent at thermoneutrality, and therefore calls into question the true nature of this knockout animal. This group rectifies this by saying that under room temperature conditions, mice eat approximately 50% more food to cope with the chronic stress of

maintaining core temperature, and UCP1 knockout mice simply generate and dissipate energy in a different manner, i.e. shivering thermogenesis is compensating for the brown adipose tissue defect. In contrast, under thermo-neutral conditions, no heat is needed and the brown adipose defects come to light. However, at thermoneutrality brown adipose tissue is inactive, and therefore a defect in brown adipose tissue function should have a net zero effect on energy expenditure and weight gain; This highlights again the need for a more thorough evaluation of these knockout mice.

Despite these findings and potential caveats, many papers have been published demonstrating the beneficial metabolic effects of brown and beige adipose tissue. Many observations published have been with whole body transgenic animals or knockout animals similar to the UCP1 knockouts described above. These reports have however caveats that remain mostly unexplored by the authors and must be critically evaluated. A novel PTEN transgenic mouse, in which PTEN was overexpressed in every tissue of the body, showed increased energy expenditure and protection from hepatic steatosis; much of which was correlated to increased brown adipose activity (16). However, one must remember that overexpression of PTEN will block insulin signaling in *all* tissues, including the liver, and in turn will prevent hepatic steatosis regardless of other metabolic alterations.

More fruitful identification of the true metabolically beneficial effects of brown or beige adipose tissue has come from tissue specific alterations in the genes which regulate adipose tissue function. The precise benefits of increasing uncoupled thermogenesis in specific organs has been explored. In a similar but more robust manner than the initial Kozak group paper, Seale et al. demonstrated that overexpression of PRDM16, a master transcriptional regulator of brown adipose tissue, caused a significant increase in “brown-like” cell development in the subcutaneous white depot (17). This change was correlated with a decrease in whole body weight on high fat diet challenge, which was due to an increase in energy expenditure rather than a decrease in food intake. Finally and most importantly, they

saw a dramatic improvement in both glucose tolerance and insulin sensitivity. Taken together, this data is some of the most impressive body of evidence with regard to the demonstration that specifically increasing beige adipocytes in the subcutaneous depot provides alleviation from body weight gain and HFD induced glucose intolerance.

Brown adipose activation

Cold is the primary driver of brown and beige function and much of this activity is derived from the nervous system. Denervation of brown adipose tissue dramatically decreases the ability of this organ to function, and denervation of the subcutaneous adipose depot eliminates the formation of beige adipocytes (18, 19). This result is due to a blockade of norepinephrine release from the nerve terminals at each of these locations. In response to norephinpherine (an afferent signal from the central nervous system, specifically the hypothalamus), β-adrenergic receptors on brown adipocytes are activated. These in turn generate cAMP resulting in the activation of PKA, which propagates multiple signals within brown adipocytes responsible for the coordinated thermogenic response of this tissue (20, 21). Activation of PKA leads to downstream activation of the transcription factors CREB, SRC and p38 (22-24). These transcription factors in turn drive other transcription factors including PGC1α causing mitochondrial biogenesis and increased thermogenesis.

An extremely important action of PKA is to increase intracellular lipolysis, an important intracellular component of this cascade required for the activation of brown adipose tissue (25). These fatty acids are able to outcompete purine nucleotides which are inhibitors of uncoupling proteins to cause proton flux through UCP proteins, regardless of the ability for these lipids to be utilized as a fuel source (26). Interestingly, recent work has demonstrated that the release of long chain fatty acids from the inner mitochondrial membrane via phospholipase A2 is required for the proper activation of these proton channels (27).

Mediators of browning

As mentioned above, a primary mediator of the brown adipose depot is β-adrenergic signaling emanating centrally from the hypothalamus primarily in response to exposure to cold conditions. Recently efforts have been looking to other endogenous signals that can regulate the amount or activity of beige and brown adipocytes. While many proteins have been found to increase the recruitment of beige precursors including meteorin, Slit2, FGF21 and IL-33, very few proteins have been found that increase the activity or cell number of the endogenous brown depot (28-30). Recently it was reported that the IL-6, a protein derived from the brown adipose depot itself, was capable of enhancing brown adipose tissue function.

IL-6 and brown adipose

In 2013 Stanford et al. published a study in which they transplanted brown adipose tissue from wildtype mice into healthy recipient and high fat diet fed recipient mice (31). In each case, the transplantation of brown adipose into recipient mice led to improvements in glucose homeostasis and insulin sensitivity. They went on to show that brown adipose tissue transplantation increased circulating IL-6 and that IL-6 knockout mice did not benefit from brown adipose tissue transplantation. They also demonstrated that transplantation of IL-6 knockout BAT did not alter body or fat pad weights. This work set the stage for IL-6 to be the first true "BATokine", responsible for its own endogenous activity in an autocrine manner. Interestingly, little follow up work was done on this.

Amlexanox

In an unexpected set of experiments, Reilly et al. demonstrated that IKKε is upregulated in the subcutaneous adipose depot of mice fed high fat diet (32). Under the assumption that increases in this inflammatory mediator were unhealthy, they used the previously FDA approved drug Amlexnaox to

inhibit its function *in vivo*. After gavaging this drug into HFD fed mice, they found that circulating IL-6 levels were dramatically upregulated in serum, and that all of this IL-6 was coming from the subcutaneous adipocytes themselves. They went on to show that these mice displayed a number of interesting metabolic improvements including decrease body weight, increased glucose tolerance and most importantly, improved activity of brown adipose tissue in high fat diet fed mice.

References

1. Rothwell NJ, Stock MJ. A role for brown adipose tissue in diet-induced thermogenesis. Nature. 1979;281(5726):31-5. Epub 1979/09/06. PubMed PMID: 551265.
2. Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nature medicine. 2013;19(10):1338-44. Epub 2013/09/03. doi: 10.1038/nm.3324. PubMed PMID: 23995282; PubMed Central PMCID: PMC4075943.
3. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. Nature. 2008;454(7207):961-7. Epub 2008/08/23. doi: 10.1038/nature07182. PubMed PMID: 18719582; PubMed Central PMCID: PMC2583329.
4. Harms MJ, Ishibashi J, Wang W, Lim HW, Goyama S, Sato T, et al. Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. Cell metabolism. 2014;19(4):593-604. Epub 2014/04/08. doi: 10.1016/j.cmet.2014.03.007. PubMed PMID: 24703692; PubMed Central PMCID: PMC4012340.
5. Berry R, Rodeheffer MS. Characterization of the adipocyte cellular lineage in vivo. Nature cell biology. 2013;15(3):302-8. Epub 2013/02/26. doi: 10.1038/ncb2696. PubMed PMID: 23434825; PubMed Central PMCID: PMC3721064.
6. Rodeheffer MS, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. Cell. 2008;135(2):240-9. Epub 2008/10/07. doi: 10.1016/j.cell.2008.09.036. PubMed PMID: 18835024.
7. Lee YH, Petkova AP, Mottillo EP, Granneman JG. In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. Cell metabolism. 2012;15(4):480-91. Epub 2012/04/10. doi: 10.1016/j.cmet.2012.03.009. PubMed PMID: 22482730; PubMed Central PMCID: PMC3322390.
8. Lepper C, Fan CM. Inducible lineage tracing of Pax7-descendant cells reveals embryonic origin of adult satellite cells. Genesis. 2010;48(7):424-36. Epub 2010/07/20. doi: 10.1002/dvg.20630. PubMed PMID: 20641127; PubMed Central PMCID: PMC3113517.
9. Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(11):4401-6. Epub 2007/03/16. doi: 10.1073/pnas.0610615104. PubMed PMID: 17360536; PubMed Central PMCID: PMC1810328.
10. Souza SC, Christoffolete MA, Ribeiro MO, Miyoshi H, Strissel KJ, Stancheva ZS, et al. Perilipin regulates the thermogenic actions of norepinephrine in brown adipose tissue. Journal of lipid research. 2007;48(6):1273-9. Epub 2007/04/03. doi: 10.1194/jlr.M700047-JLR200. PubMed PMID: 17401109.
11. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell. 2012;150(2):366-76. Epub 2012/07/17. doi: 10.1016/j.cell.2012.05.016. PubMed PMID: 22796012; PubMed Central PMCID: PMC3402601.
12. Wang W, Kissig M, Rajakumari S, Huang L, Lim HW, Won KJ, et al. Ebf2 is a selective marker of brown and beige adipogenic precursor cells. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(40):14466-71. Epub 2014/09/10. doi: 10.1073/pnas.1412685111. PubMed PMID: 25197048; PubMed Central PMCID: PMC4209986.
13. Kopecky J, Clarke G, Enerback S, Spiegelman B, Kozak LP. Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. The Journal of clinical investigation. 1995;96(6):2914-23. Epub 1995/12/01. doi: 10.1172/JCI118363. PubMed PMID: 8675663; PubMed Central PMCID: PMC186003.
14. Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, et al. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature. 1997;387(6628):90-4. Epub 1997/05/01. doi: 10.1038/387090a0. PubMed PMID: 9139827.

15. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell metabolism. 2009;9(2):203-9. Epub 2009/02/04. doi: 10.1016/j.cmet.2008.12.014. PubMed PMID: 19187776.
16. Ortega-Molina A, Efeyan A, Lopez-Guadamillas E, Munoz-Martin M, Gomez-Lopez G, Canamero M, et al. Pten positively regulates brown adipose function, energy expenditure, and longevity. Cell metabolism. 2012;15(3):382-94. Epub 2012/03/13. doi: 10.1016/j.cmet.2012.02.001. PubMed PMID: 22405073.
17. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. The Journal of clinical investigation. 2011;121(1):96-105. Epub 2010/12/03. doi: 10.1172/JCI44271. PubMed PMID: 21123942; PubMed Central PMCID: PMC3007155.
18. Desautels M, Dulos RA, Mozaffari B. Selective loss of uncoupling protein from mitochondria of surgically denervated brown adipose tissue of cold-acclimated mice. Biochemistry and cell biology = Biochimie et biologie cellulaire. 1986;64(11):1125-34. Epub 1986/11/01. PubMed PMID: 3828106.
19. Pulinilkunnil T, He H, Kong D, Asakura K, Peroni OD, Lee A, et al. Adrenergic regulation of AMP-activated protein kinase in brown adipose tissue in vivo. The Journal of biological chemistry. 2011;286(11):8798-809. Epub 2011/01/07. doi: 10.1074/jbc.M111.218719. PubMed PMID: 21209093; PubMed Central PMCID: PMC3059037.
20. Fredriksson JM, Thonberg H, Ohlson KB, Ohba K, Cannon B, Nedergaard J. Analysis of inhibition by H89 of UCP1 gene expression and thermogenesis indicates protein kinase A mediation of beta(3)-adrenergic signalling rather than beta(3)-adrenoceptor antagonism by H89. Biochimica et biophysica acta. 2001;1538(2-3):206-17. Epub 2001/05/05. PubMed PMID: 11336791.
21. Zhao J, Cannon B, Nedergaard J. alpha1-Adrenergic stimulation potentiates the thermogenic action of beta3-adrenoreceptor-generated cAMP in brown fat cells. The Journal of biological chemistry. 1997;272(52):32847-56. Epub 1998/01/31. PubMed PMID: 9407062.
22. Thonberg H, Fredriksson JM, Nedergaard J, Cannon B. A novel pathway for adrenergic stimulation of cAMP-response-element-binding protein (CREB) phosphorylation: mediation via alpha1-adrenoceptors and protein kinase C activation. The Biochemical journal. 2002;364(Pt 1):73-9. Epub 2002/05/04. PubMed PMID: 11988078; PubMed Central PMCID: PMC1222547.
23. Lindquist JM, Fredriksson JM, Rehnmark S, Cannon B, Nedergaard J. Beta 3- and alpha1-adrenergic Erk1/2 activation is Src- but not Gi-mediated in Brown adipocytes. The Journal of biological chemistry. 2000;275(30):22670-7. Epub 2000/04/20. doi: 10.1074/jbc.M909093199. PubMed PMID: 10770951.
24. Cao W, Medvedev AV, Daniel KW, Collins S. beta-Adrenergic activation of p38 MAP kinase in adipocytes: cAMP induction of the uncoupling protein 1 (UCP1) gene requires p38 MAP kinase. The Journal of biological chemistry. 2001;276(29):27077-82. Epub 2001/05/23. doi: 10.1074/jbc.M101049200. PubMed PMID: 11369767.
25. Nicholls DG. The physiological regulation of uncoupling proteins. Biochimica et biophysica acta. 2006;1757(5-6):459-66. Epub 2006/05/27. doi: 10.1016/j.bbabio.2006.02.005. PubMed PMID: 16725104.
26. Shabalina IG, Backlund EC, Bar-Tana J, Cannon B, Nedergaard J. Within brown-fat cells, UCP1-mediated fatty acid-induced uncoupling is independent of fatty acid metabolism. Biochimica et biophysica acta. 2008;1777(7-8):642-50. Epub 2008/05/21. doi: 10.1016/j.bbabio.2008.04.038. PubMed PMID: 18489899.
27. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. Cell. 2012;151(2):400-13. Epub 2012/10/16. doi: 10.1016/j.cell.2012.09.010. PubMed PMID: 23063128; PubMed Central PMCID: PMC3782081.

28. Van den Berg TK, Dopp EA, Breve JJ, Kraal G, Dijkstra CD. The heterogeneity of the reticulum of rat peripheral lymphoid organs identified by monoclonal antibodies. European journal of immunology. 1989;19(9):1747-56. Epub 1989/09/01. doi: 10.1002/eji.1830190933. PubMed PMID: 2676562.
29. Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. Cell. 2014;157(6):1279-91. Epub 2014/06/07. doi: 10.1016/j.cell.2014.03.065. PubMed PMID: 24906147; PubMed Central PMCID: PMC4131287.
30. Lee MW, Odegaard JI, Mukundan L, Qiu Y, Molofsky AB, Nussbaum JC, et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. Cell. 2015;160(1-2):74-87. Epub 2014/12/30. doi: 10.1016/j.cell.2014.12.011. PubMed PMID: 25543153; PubMed Central PMCID: PMC4297518.
31. Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. The Journal of clinical investigation. 2013;123(1):215-23. Epub 2012/12/12. doi: 10.1172/JCI62308. PubMed PMID: 23221344; PubMed Central PMCID: PMC3533266.
32. Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, et al. An inhibitor of the protein kinases TBK1 and IKK-varepsilon improves obesity-related metabolic dysfunctions in mice. Nature medicine. 2013;19(3):313-21. Epub 2013/02/12. doi: 10.1038/nm.3082. PubMed PMID: 23396211; PubMed Central PMCID: PMC3594079.

Chapter 4

Amlexanox Reverses High Fat Diet Induced Adrenergic Resistance In an IL-6 Dependent Manner

(The following is part of a manuscript pending submission)

Thomas S. Morley[1], Joshua Johnson[1], Alexandra Ghaben[1], William L. Holland[1], Thomas Wunderlich[2], Jens Bruening[2] and Philipp E. Scherer[1,*]

[1]Touchstone Diabetes Center, Department of Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, Texas 75390-8549.

[2]Department of Neuronal Control of Metabolism, Max Planck Institute for Metabolism Research, Cologne, Germany

[*]Correspondence should be addressed to:
Philipp E. Scherer
Touchstone Diabetes Center
Department of Internal Medicine
University of Texas Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX, 75390-8549, USA
E-mail: Philipp.Scherer@utsouthwestern.edu
Tel: 214-648-8715
Fax: 214-648-8720

Abstract

Increased caloric intake and the resulting obesity are responsible for a number of physiological changes, most notably insulin resistance in many peripheral tissues including muscle and adipose tissue (1). Interleukin 6 (IL-6) is among a group of inflammatory cytokines elevated in adipose tissue during states of obesity and its levels correlate with decreased insulin sensitivity in adult humans (2). Here we use an inducible knock-out model for the IL-6 receptor in adipocytes to demonstrate that IL-6 is not a mediator of adipose tissue dysfunction during HFD feeding in rodents. Furthermore, much attention has been given to therapies that regulate brown or beige adipose tissue function (3). We demonstrate here that the new anti-obesity and anti-diabetic IKKβ inhibitor Amlexanox triggers lasting metabolic improvements in mice within as little as 2 weeks of exposure that persist after the inhibitor treatment is stopped. Amlexanox treatment leads to a high level induction of IL-6, and it is the IL-6 action directly on the adipocyte that mediates the beneficial effects. We conclude that Amlexanox triggers a persistent enhancement of the sensitivity of adipose tissue to β- adrenergic signaling.

Introduction

Diabetes and insulin resistance remain highly detrimental diseases in terms of both morbidity and mortality. However the underlying mechanisms responsible for these processes remain poorly understood. During states of high fat diet-induced obesity, adipose tissue becomes inflamed. IL-6 levels in adipose tissue rise in this setting and IL-6 levels in serum are inversely correlated with systemic insulin sensitivity (2). Despite these strong correlations, the role of IL-6 in metabolism has been a widely debated topic for much of the past two decades, and it remains unclear whether we should view this cytokine as a beneficial or detrimental factor.

In vitro, many groups have demonstrated that IL-6 induces insulin resistance in a number of cultured cell lines, including hepatocytes and skeletal muscle, though in the latter, a brief exposure enhanced glucose uptake while chronic exposure decreased it (4, 5). This data is further corroborated *in vivo* via overexpression of IL-6 by skeletal muscle which resulted in dramatic hyperinsulinemia and reduced glucose uptake in muscle (6). Others have shown that chronic administration of recombinant IL-6 aggravated already increased steatosis in IL-6 knockout mice, a paradoxical yet interesting result (7). Most recently, others have shown that blocking IL-6 signaling decreased adipose macrophage recruitment in the setting of a high fat diet challenge, but surprisingly, this did not result in improved insulin sensitivity (8). In contrast to these studies is work showing that IL-6 knockout mice develop diabetes, implying that IL-6 may be necessary to maintain glucose homeostasis (9). With dichotomous results such as these, more effort is required to fully understand the role of IL-6 in metabolism. In an effort to

understand the role of IL-6 signaling in the adipocyte more mechanistically, we have engineered a mouse with an inducible elimination of the IL-6 receptor from mature adipocytes. To do that, we employ a combination of previously verified transgenic models, including an adiponectin promoter driven reverse tetracycline trans activator transgenic mouse, crossed with a tetracycline response element driven CRE recombinase transgenic line, both in a homozygous floxed IL-6 receptor α background (10, 11). Upon administration of doxycycline-containing diet, the IL-6 receptor is excised from all mature adipocytes expressing adiponectin. Using this model, we show that this mouse is metabolically normal and shows no alterations in glucose tolerance or insulin sensitivity *in vivo*. These results are surprising and indicate that metabolic dysfunction arising during high fat diet feeding is not a result of IL-6 action on the adipocyte itself.

Recently, the drug Amlexanox has created a stir in the field of metabolic research for its potent ability to cause weight loss in obese animals combined with its highly anti-steatotic action (12). Amlexanox induces very high levels of IL-6 in the serum of high fat diet fed mice, and this IL-6 originates primarily from the subcutaneous adipocytes themselves (12). A recent publication has linked the improvements in hepatic function seen when mice are given Amlexanox to direct action of IL-6 on the liver (13). This drug provides a mechanism by which circulating IL-6 levels can be dramatically increased without the use of recombinant forms of this cytokine. It also provides a significant improvement in overall metabolic health. We therefore decided to take advantage of Amlexanox and its downstream action to explore its mechanism of action in the context of the adipocyte-specific IL-6R knockout. Our findings indicate that the improvements in glucose tolerance seen with Amlexanox treatment rely entirely on its ability of the drug to

induce weight loss. Remarkably, improvements in hepatic steatosis and brown adipose lipid oxidation are extraordinarily durable, and the effects on these tissues persist long after Amlexanox exposure has stopped. Using our inducible knockout animals lacking IL-6 receptor in the adipocyte, we show that these improvements are primarily due to direct IL-6 action on adipocytes. Underlying these improvements is an enhanced response in adipose depots to β adrenergic signaling, resulting in decreased subcutaneous adipose weights and increased brown adipose activity. Overall our data demonstrates that the long term metabolic benefits seen with Amlexanox involve a direct autocrine action of IL-6 on adipose depots.

Results

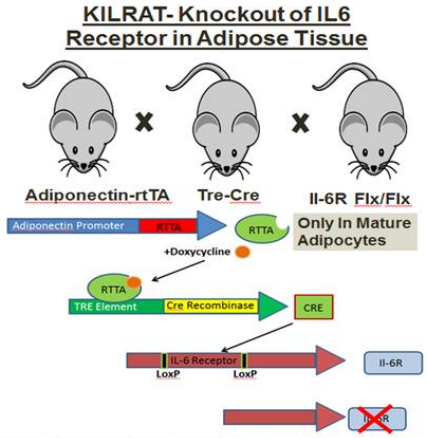


Fig1A. Schematic View for generation of inducible deletion of the IL6-receptor from adipocytes

As increases in IL-6 are concomitant with HFD feeding and insulin resistance in white adipose tissue, we wished to address whether this IL-6 induction directly alters adipose tissue insulin sensitivity and glucose tolerance. To this end, we generated a model in which the IL-6 receptor α can be inducibly deleted from mature white adipocytes. Using an adiponectin promoter driven reverse tetracycline transactivator transgenic mouse, coupled with a tetracycline response element driven CRE recombinase transgenic line, in a homozygous floxed IL-6 receptor background (referred to as the KILRAT mouse), we were able to inducibly delete the IL-6 specifically in adipocytes upon exposure to doxycycline (**Fig. 1A**). The adiponectin driven cre system has previously been verified by Morley et al. (26243466) while the floxed IL-6 receptor line has previously been verified by (24681566). To confirm this system works in our hands, 6 week old male mice were placed for 4 weeks on chow diet containing 600mg/kg doxycycline. Mice were sacrificed and adipose depots and control tissues extracted and analyzed for the presence of the IL-6 receptor via RNA extraction, cDNA synthesis and RTqPCR analyses; control mice were either adipo-rtTA or TRE-CRE negative. **Fig. 1B** shows the relative expression of this receptor and confirms our model is effectively eliminating the IL-6 receptor from the subcutaneous and gonadal adipose depots (**Fig1B**).

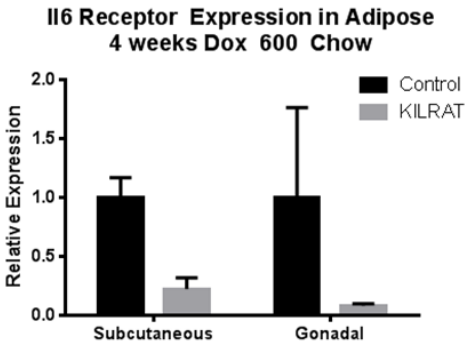


Fig1B- Relative expression of IL6 Receptor in adipose depots of Mice exposed to Doxycycline containing diet (600mg/kg) for 4 weeks

To determine if the loss of the IL-6 receptor from adipocytes has any metabolic consequences on a chow diet, we placed 6 week old male mice on a chow diet containing 600mg/kg doxycycline. After 4 weeks, these mice were challenged with an oral glucose tolerance test, which demonstrated no difference between the KILRAT or control cohorts (**Fig. 1C**).

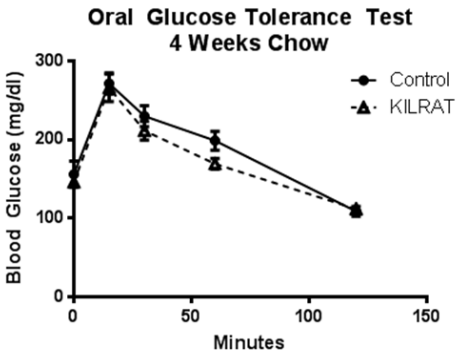


Fig1C- Blood glucose levels of control and KILRAT mice during OGTT following 4 weeks of chow diet containing Doxycycline (600mg/kg) (n=6 control and 4 KILRAT)

As IL-6 has been implicated in the metabolic dysregulation occurring upon HFD-induced obesity, we decided to challenge the KILRAT mice with high fat diet containing doxycycline (600mg/kg).

Following 6 weeks of HFD feeding, oral glucose tolerance tests were performed in which mice were fasted for 4 hours and then gastric gavage was performed with D-glucose at a dose of 2.5g/kg (**Fig.2a, b**). Again, these experiments failed to demonstrate any differences in glucose tolerance or insulin secretion. At 8 weeks of age, we subjected these mice to direct insulin sensitivity testing using an insulin tolerance test (**Fig, 2c**). Mice were fasted for 4 hours and then injected with Humilin R recombinant insulin at a dose of 1IU/kg body weight. These tests demonstrated equivalent insulin sensitivity between the 2 groups. Mice

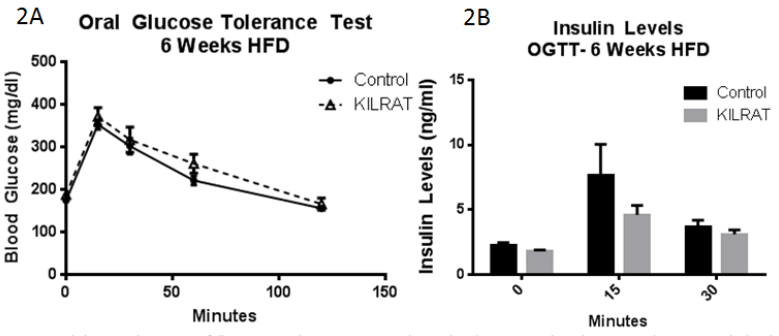


Fig2B- Oral glucose tolerance test following 6 weeks HFD containing doxycycline (N=14 controls and 10 KILRATS). Fig 2B- Insulin levels from randomly selected mice during oral glucose tolerance test shown in Fig2A (N=4 each group)

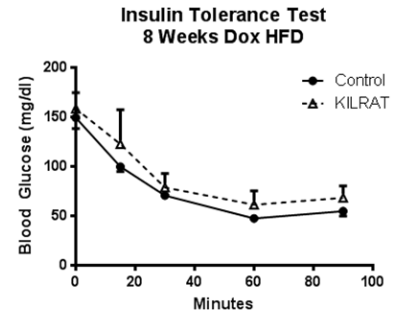


Fig2C- Insulin tolerance test performed on control and KILRAT mice after 8 weeks HFD containing doxycycline (600mg/kg) (N=5 control and 4 KILRAT

were again tested for glucose tolerance at 12 weeks following the initiation of HFD (**Fig. 2D, E**). Again, the two groups showed no differences in glucose tolerance or insulin secretion. Body weight curves of the mice over the 12 week time period revealed no significant differences, leading to the conclusion that in these mice, IL-6 signaling in the adipocyte does not seem to matter from a metabolic perspective (**Fig. 2F**).

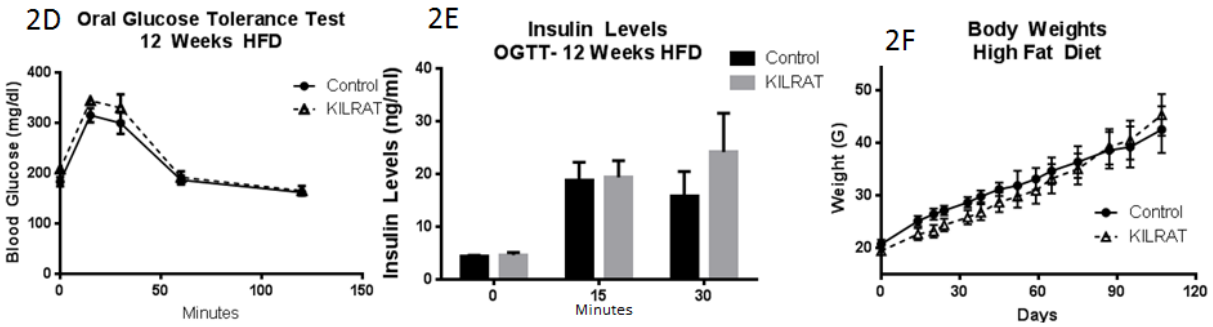


Fig2D- Oral glucose tolerance test following 12 weeks HFD containing doxycycline (N=7 controls and 5 KILRATS). Fig 2E- Insulin levels from randomly selected mice during oral glucose tolerance test shown in Fig2D (N=4 each group) Fig2F- Body weights of Control and KILRAT mice on high fat diet containing doxycycline at 600mg/kg (N=5 control and 4 KILRAT)

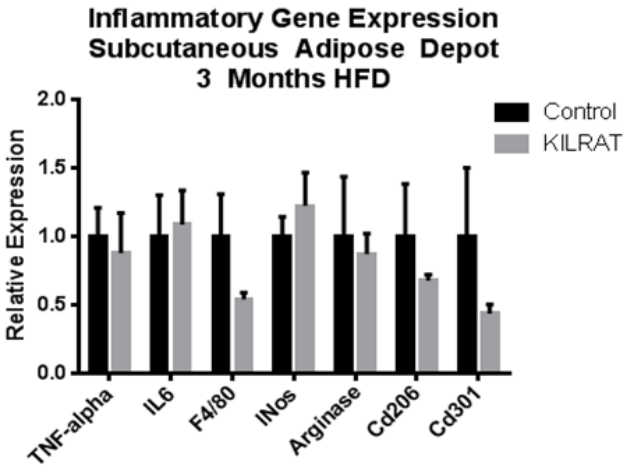


Fig2G- Inflammatory gene expression in subcutaneous adipose depot of Control and KILRAT mice exposed to HFD containing doxycycline for 12 weeks (N=3 each group)

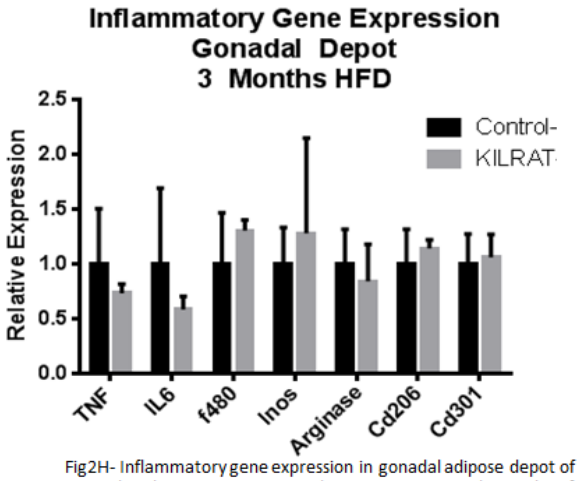


Fig2H- Inflammatory gene expression in gonadal adipose depot of Control and KILRAT mice exposed to HFD containing doxycycline for 12 weeks (N=3 each group)

To better understand the inflammatory consequences following the loss of the IL-6 receptor from adipocytes, we isolated both the subcutaneous and gonadal adipose depots from 12 week old HFD fed KILRAT and control mice. RNA was isolated, cDNA synthesized and qPCR analysis performed (**Fig. 2G+2H**). This analysis showed no significant differences between the groups,

though the subcutaneous adipose depot did show a trend towards decreased macrophage infiltration. Overall these data suggest the adipocyte autocrine signaling is not a target of IL-6 mediated metabolic dysfunction during dietary induced obesity.

Amlexanox

Classically, elevations in circulating IL-6 have been associated with detrimental metabolic outcomes, including elevated circulating non-esterified fatty acids and decreased insulin sensitivity (2, 14). Recently however, a series of papers have demonstrated that the IKKβ inhibitor amlexanox is capable of dramatically elevating IL-6 levels *in vivo* (12). Furthermore, these reports describe the dramatic improvements metabolic homeostasis seen upon a HFD challenge as an IL-6 dependent phenomenon, since a neutralizing IL-6 antibody was able to inhibit the positive metabolic benefits brought about by amlexanox (13). To test the effects of amelxanox and IL-6 on adipose tissue, we wished to determine whether we could reproduce the beneficial metabolic effects previously reported.

To do that, we placed 6 week old wildtype C57Bl6 mice on high fat diet for 12 weeks. These mice were then gavaged daily with amlexanox at 50mg/kg, a dose previously reported to potently induce IL-6. Four hours post gavage, blood was sampled and serum analyzed via ELISA, indeed revealing dramatically elevated levels of circulating IL-6 in the treated group (**Fig. 3a**). Mice were continually treated daily with amlexanox or control solution (described in Materials and Methods) for 2 weeks. At this time point, a metabolic analysis was

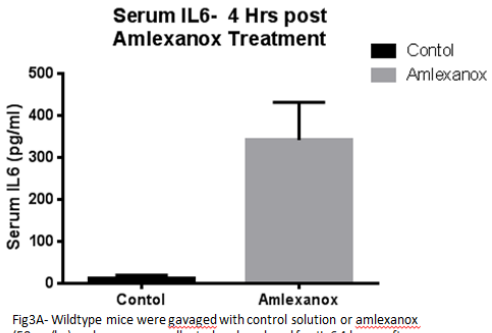


Fig3A- Wildtype mice were gavaged with control solution or amlexanox (50mg/kg) and serum was collected and analyzed for IL-6 4 hours after the gavage (n=4 each group)

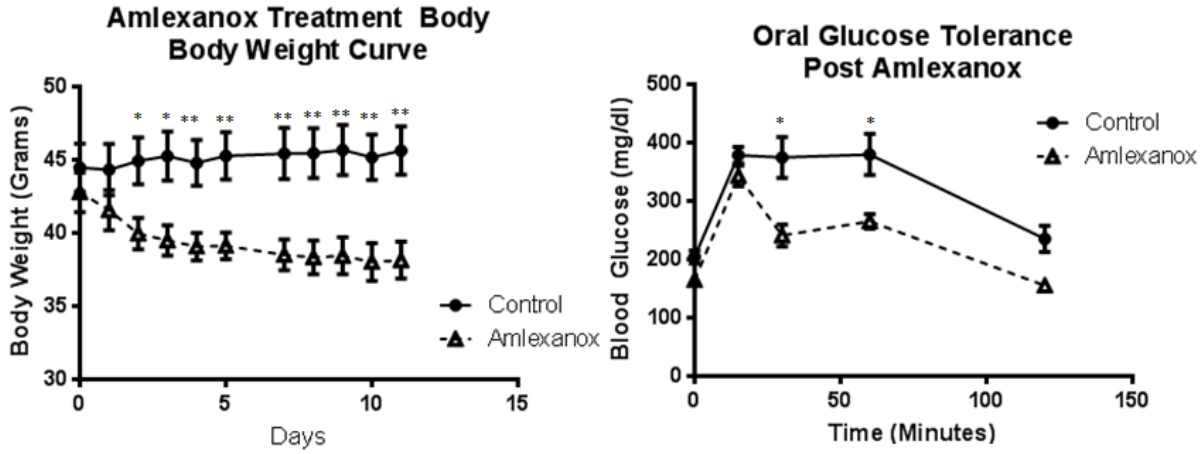


Fig3B- Bodyweights of mice gavaged with Amlexanox or control solution for 12 days (n=6 each group) Fib3C- Oral glucose tolerance test of mice 2 days after cessation of Amlexanox therapy (n=6 and 6 for both groups)

performed as the body weights had diverged significantly between treatment and control groups (**Fig. 3B**). An oral glucose tolerance test revealed dramatic improvements between the treated and untreated groups (**Fig. 3C**). As Amlexanox treatment corresponded with both improvements in oral glucose tolerance and decreases in body weights, we wanted to assess the effects of this drug on improving brown or beige adipose function, as had previously been reported (cite). To this end, we subjected mice to a radioactive triolein chase experiment. Briefly, radioactive triolein is incorporated into lipid micelles and injected into the tail vein of unanesthetized mice; clearance is monitored via tail vein bleeds and after 15 minutes mice are sacrificed and tissues taken to determine lipid uptake and oxidation rates. Our triolein chase demonstrated a dramatic

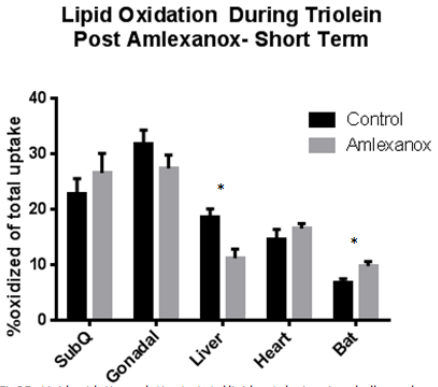


Fig3D- Lipid oxidation relative to total lipid uptake in mice challenged with IV triolein treatment following a 4 hours fast (n=4 each group)

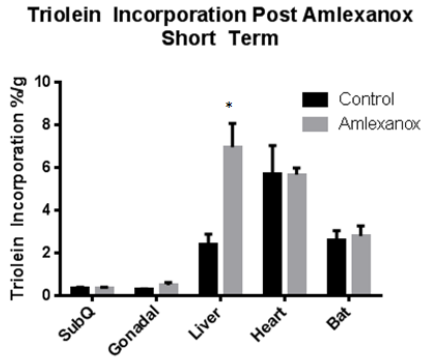


Fig3E- Total radioactive lipid incorporation per tissue as percent of total injection per gram tissue (n=4 each group)

improvement in lipid oxidation in the brown adipose depot upon Amlexanox exposure (**Fig. 3D**). The livers of the Amlexanox treated mice were oxidizing less lipid as a percent of the total lipid taken up by the liver. However, the Amlexanox treated mice had overall a dramatic increase in hepatic lipid uptake (**Fig. 3E**). Very unexpectedly, we found that the subcutaneous adipose depots were dramatically reduced in size in the Amlexanox treatment group (**Fig. 3F**). This was in contrast to the gonadal adipose depots which showed no alteration in size.

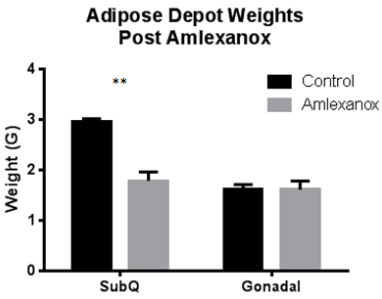


Fig3F- Adipose depot weights following amlexanox treatment (n=5 control and 4 amlexanox)

To understand the mechanistic changes underlying these dramatic improvements, gene expression analysis was performed on the subcutaneous and brown adipose depots (**Fig. 3G**). In the subcutaneous depots, we found a significant increase in hormone sensitive lipase (HSL) mRNA as well as a trend toward significant increases in adipose triglyceride lipase (ATGL) (p=0.036 and 0.099 respectively). Further, we saw a decrease in Elovl-3 message (p= 0.058), though no major alterations in any other known brown or beiging genes were observed. In brown adipose, we saw no significant changes in brown adipose specific transcripts (**Fig. 3H)**

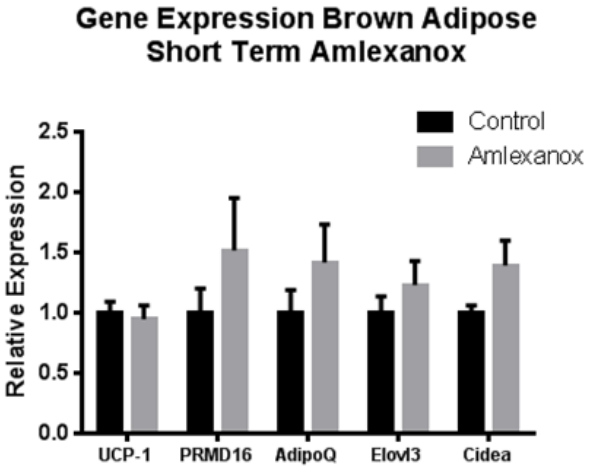


Fig3H- Quantitative PCR analysis revealing gene expression alterations in brown adipose depot one week following amlexanox treatment (n=4 and 4)

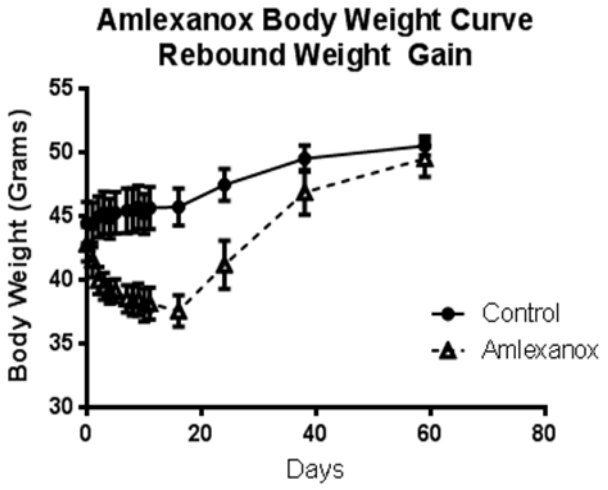


Fig 3I- Body weights of mice treated with Amlexanox for two weeks then maintained on High Fat diet for another 6 weeks

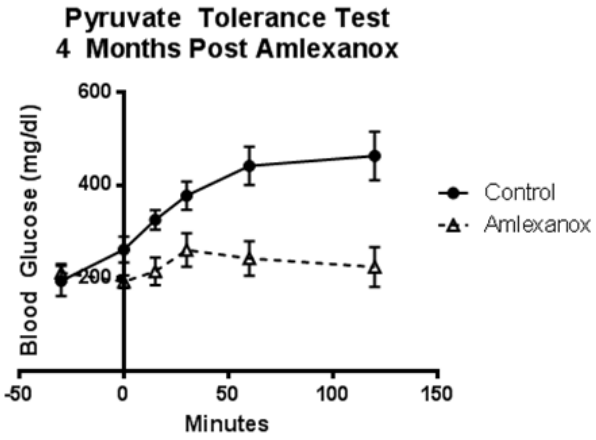


Fig 3K- Blood glucose levels of mice subjected to a pyruvate tolerance test (2g/kg)

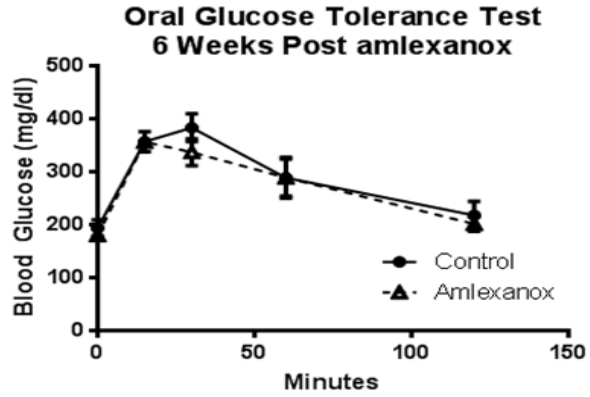


Fig3J- blood glucose values of mice subjected to oral glucose tolerance testing 6 weeks following cessation of Amlexanox treatment (n=6 for each group)

The rapid changes in glucose tolerance were impressive, however they were associated with multiple alterations in organismal physiology, including altered levels of message for multiple adipose genes in the white adipose and significant changes in body weight. As glucose tolerance is dramatically affected by the weight of the mice, we wished to delineate which effects rely on brown adipose tissue and which were body weight dependent. To this end, a cohort of wild type mice were placed on high fat diet at 6 weeks of age; after 8 weeks they were subjected to daily amlexanox gavages at 50mg/kg for 2 weeks. After amlexanox exposure, these mice weighed significantly less and they were monitored longitudinally for rebound weight gain (**Fig. 3I**). After 8 weeks, body weight differences between the 2 groups were no longer significantly different, and metabolic testing was initiated. An oral glucose tolerance test was performed, and we found no significant differences between the two groups (**Fig. 3J**). This data suggests that the improvements seen in glucose tolerance following amlexanox exposure are primarily due to decreases in body weight.

At these long times points following treatment, we wondered if other aspects of their treatment were more durable. One of the main end points of the initial papers published about

Amlexanox had been an improved pyruvate tolerance test. To test whether this was an effect that could be seen at later time points, mice who had been treated in a similar manner to those in **Fig. 3J**, but were 4 months *post* from their last dose of Amlexanox, were subjected to a pyruvate tolerance test. Surprisingly, the mice treated with Amlexanox handled the pyruvate tolerance test very well, while the mice that had been gavaged with vehicle, had extremely high blood glucose levels reflecting insulin resistance and elevated gluconeogenic potential in their livers (**Fig. 3K**).

These observations combined are consistent with a model in which improved β adrenergic sensitivity in adipose depots is driving the phenotype, causing increased lipid oxidation and activation of brown adipose tissue, as well as enhanced lipolysis in subcutaneous depots, leading to decreased depot weights. Enhanced brown adipose function has been shown to reduce hepatic steatosis and restore proper liver function, again, a possible mechanism explaining the improvements seen during the pyruvate tolerance test. Therefore, mice shown in **Fig. 3J** that had overlapping oral glucose tolerance curves were subjected to a β adrenergic stimulation test. Mice were fasted for 4 hours and then injected with a low dose of the β3 receptor agonist CL316 243 (**Fig. 3L**). What we found is that those mice which had been treated with amlexanox had elevated NEFA levels in their blood compared ot those mice which had been control gavaged following β3 receptor stimulation.

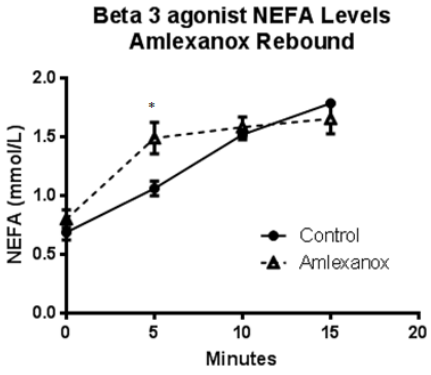


Fig 3L-NEFA values following low dose beta-3 adrenergic stimulation with CL316,243 following 4 hour fast (n=4 control and 5 amlexanox)

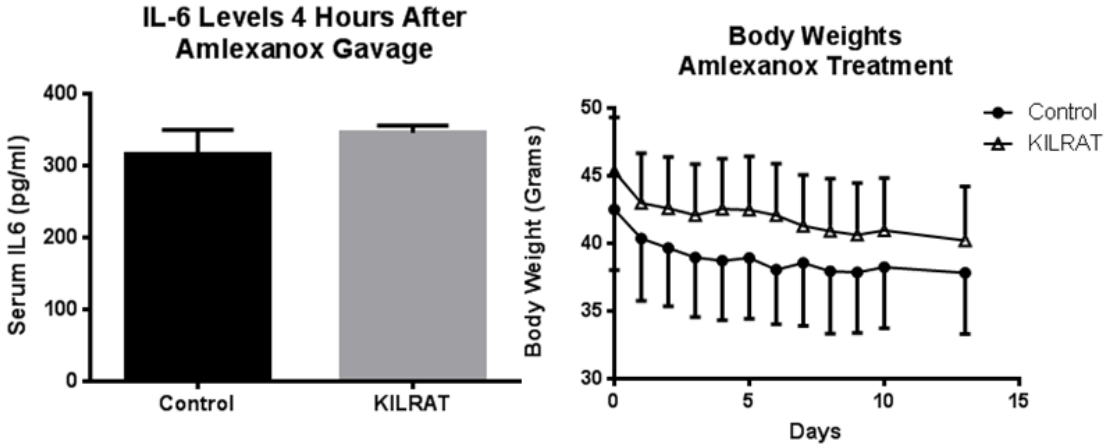


Fig4A-serum IL6 levels following amlexanox gavage in control or KILRAT mice (n=7 control and 5 KILRAT). Fig B- Body weights of control and KILRAT mice following daily amlexanox gavages (50mg/kg) (n=5 control and 4 KILRAT)

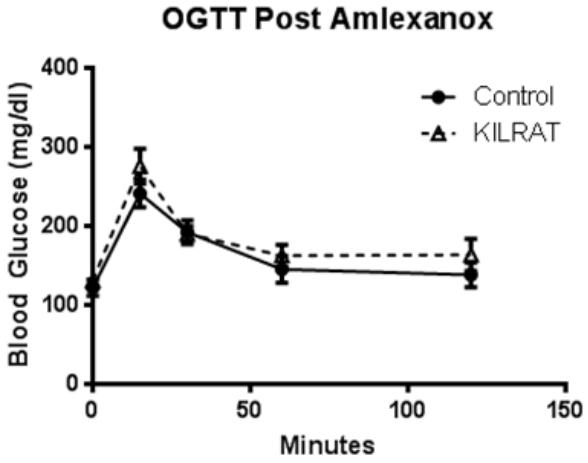
KILRAT Mice treated with amlexanox

In previous publications looking at the beneficial metabolic effects of amlexanox, a neutralizing IL-6 antibody was shown to neutralize the actions of this compound. As we observed dramatic alterations in subcutaneous adipose depot gene expression and overall size, as well as increases in brown adipose lipid oxidation, we hypothesized that these effects were due to IL-6 signaling directly on the adipose depots. Using the KILRAT model in which we inducibly delete the IL-6 receptor form all white and a large proportion of brown adipocytes, we wanted to address the direct role of IL-6 signaling with regard to the metabolically beneficial effects of Amlexanox.

KILRAT mice and control mice that had been exposed to a HFD containing doxycycline (600mg/kg) for 12 weeks were treated with daily oral Amlexanox gavages for 2 weeks, totaling 12 doses. To confirm that the KILRAT mice had no defect in IL-6 secretion following Amlexanox treatment, IL-6 levels were measured in serum 4 hours after the first dose of Amlexanox using an ELISA. This revealed no defect in the ability of Amlexanox to drive IL-6 production in our IL-

6R null mice (**Fig. 4A**). Over the course of 12 doses, KILRAT and control mice showed similar decreases in body weight (**Fig. 4B**).

An oral glucose tolerance test performed 2 days after the final dose of Amlexanox showed now apparent differences between the groups' responses to the treatment, and both showed lower average AUC compared to an oral glucose tolerance test performed before the initiation of the Amlexanox therapy (**Fig. 4C and 4D**).



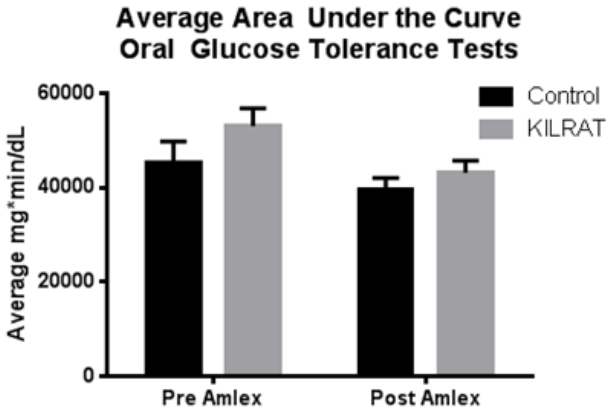


Fig4C- Blood glucose values of mice post amlexanox treatment for 2 weeks (n=5 control and 4 KILRAT) Fig 4D- Average area under the curve for control and KILRAT mice before and after a 2 week course of Amlexanox therapy (n= 5 control and 4 KILRAT)

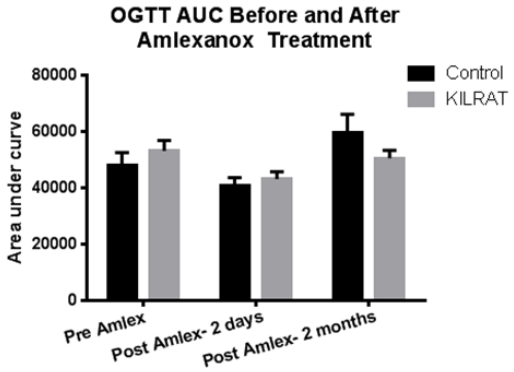


Fig 4E- Average area under the curve for control and KILRAT mice before, directly after, or 2 months after Amlexanox therapy (n=5 control and 4 KILRAT)

To determine if similarly observed durable improvements in metabolism are present in the KILRAT mice as they can be observed in the control mice, the mice shown **Fig. 4C** were allowed to regain body weight over an extended period of time, while continuing to be fed a HFD containing 600mg/kg doxycycline. After 2 months an oral glucose tolerance test was performed and showed both groups having increased glucose intolerance compared to the OGTT performed shortly after the initial Amlexanox therapy, though none of these points were statistically significant (**Fig 4E**). Interestingly, the KILRAT mice began to diverge dramatically in

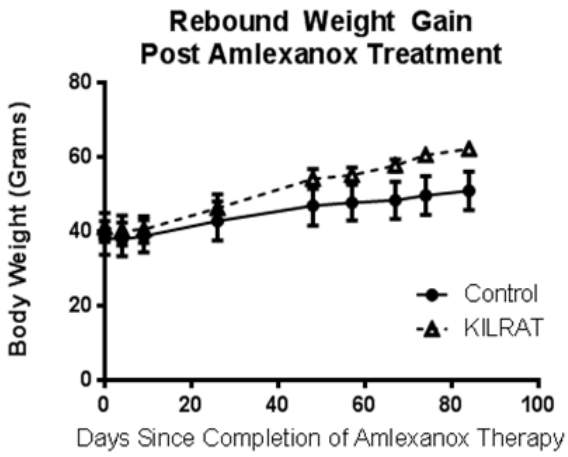


Fig4F- Body weights of Control and KILRAT mice following 2 weeks of Amlexnaox treatment fed on High Fat diet (n=5 control and 4 KILRAT)

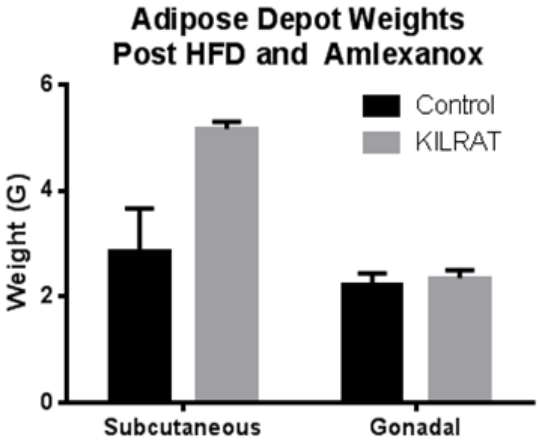


Fig 4G- Adipose depot weights of Control and KILRAT mice approximately 3 Months Post Amlexanox therapy (n=5 control and 4 KILRAT)

body weight at distant time points during weight regain, though these values did not reach statistical significance (p=0.09 at most distant point) (**Fig. 4F**). These mice were sacrficied and tissues harvested. The control mice at this very long time point had signficantly smaller subcutaneous adipose depots as compared to the KILRAT mice (**Fig. 4G**). Livers were taken for histology at this point. Suprisingly, the livers of the control mice were non-steatotic compared to those from the KILRAT mice (**Fig. 4H**).

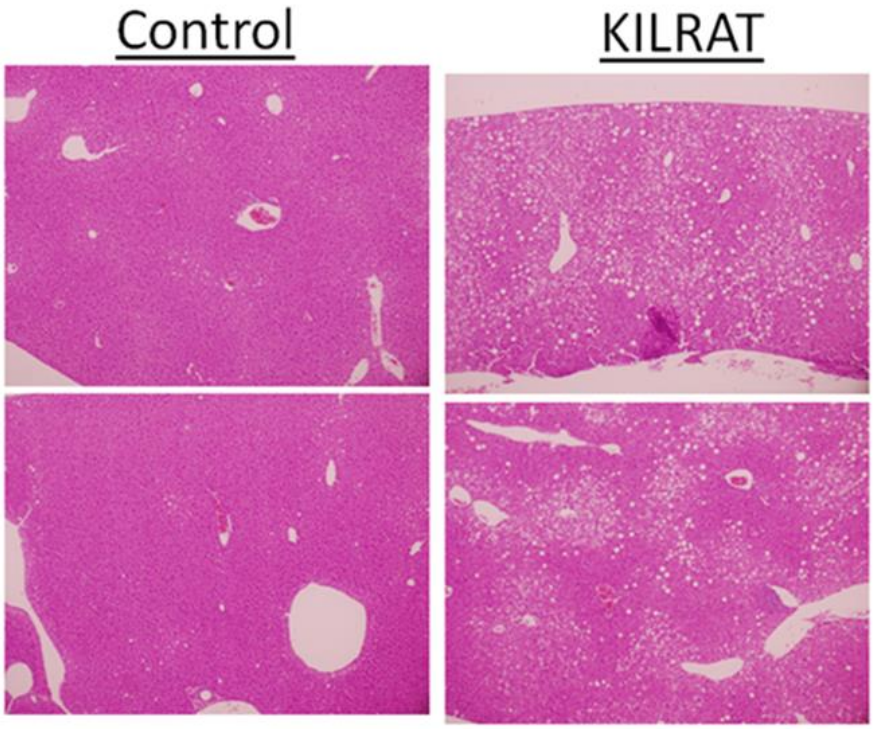


Fig4H- H+E staining of the livers of mice which had been fed high fat diet for 3 months following their 2 week course of treatment with Amlexanox

Discussion

Inflammation occurring during high fat diet feeding has been demonstrated to be causative in metabolic dysfunction (15). One of the key cytokines implicated in this dysfunction is IL-6. However, the relative importance and whether IL-6 truly mediates dysfunction remain unexplored. Herein, we demonstrate that with loss of the IL6 receptor from adipocytes, high fat diet feeding induced metabolic dysfunction is unaffected as judged by glucose tolerance and insulin sensitivity. Further, loss of this receptor did not attenuate IL-6 expression in either of the adipose depots measured under high fat diet fed conditions, nor did it alter macrophage infiltration to a significant extent. Therefore, we conclude that IL-6 does not target the adipocyte directly to mediate metabolic dysfunction during high fat diet induced obesity.

The recent studies involving the drug amlexanox as a potential therapeutic for diabetes and metabolic syndrome has created a lot of excitement, as this drug is already FDA approved to treat a number of inflammatory conditions (even though it is currently discontinued from further production). In several recent publications, it was found that this drug can lower body weights, improve glucose tolerance, decrease hepatic steatosis and enhance brown adipose gene markers (12, 13). Furthermore, this drug triggered increases in circulating IL-6 levels, while improving metabolic function system wide; a highly unexpected result given IL-6's long history of being viewed as unfavorable for glucose tolerance and insulin sensitivity. Therefore we wished to explore its function further *in vivo*.

We show that amlexanox indeed induces dramatic weight loss in rodents with pre-existing obesity, and it causes huge surges in circulating IL-6 levels. Furthermore, the weight loss triggers improvements in glucose tolerance; however this effect is lost upon rebound weight

gain. We further show that amlexanox mediates improvements in brown adipose function via direct measurements of lipid oxidation rates using radioactive tracers. Interestingly, we demonstrate that for this effect to kick in, it takes just 2 weeks of amlexanox treatment. More importantly however, we demonstrate durable changes resulting from these short-term treatments with amlexanox. Though the mice are no long more glucose tolerant at these later time points, they have a dramatically better ability to handle a pyruvate load, indicating significant decreases in gluconeogenic activity. When these changes are assessed together, along with the selective decrease in subcutaneous adipose depot weight and altered gene program, it appears that these mice are more sensitive to the endogenous β adrenergic signaling. We tested this directly and they were found to be more sensitive to β-3 adrenergic receptor activation as judged by significant increases in circulating NEFAs following an injection with CL316,324.

As these changes could be due to direct action of amlexanox, or indirectly through its ability to drive increases in circulating IL-6, we used our KILRAT mice to test the latter model. The KILRAT mice responded similarly as wildtype controls in terms of increases in circulating IL-6, decreases in body weight, and improvements in oral glucose tolerance tests. However, they differed in several important ways. Upon rebound weight gain, it was found that the subcutaneous adipose depots of the KILRAT mice were significantly larger than that of the control mice. Furthermore, the KILRAT mice had dramatic hepatic steatosis when compared with the controls. These results indicate the necessity of direct IL-6 signaling on the adipocyte proper as a mediator of the long-term benefits of Amlexanox.

Overall our observations show that IL-6 signaling in the adipocyte is the primary mediator of the long-term benefits of amlexanox treatment, while it is not a direct mediator of adipocyte metabolic dysfunction following a high fat diet.

Materials and Methods

Animals

Animal care and experimental protocols were approved by the Institutional animal care and use committee of the University of Texas Southwestern medical center. Adiponectin-RTTA mice were produced as previously described (11). Tre-Cre mice were purchased from Jackson laboratories. IL6 receptor floxed mice were produced as previously published(10). All experiments were done using male mice with littermate controls that were adiponectinP-rtTA or TRE-cre negative. Mice were fed a standard chow diet (number 5058, LabDiet), a Dox-chow diet (600 mg kg^{-1} Dox; Bio-Serv), a 60% HFD (D12492, Research Diets Inc.) or a Dox-HFD (600 mg kg^{-1} Dox; Bio-Serv). Following weaning mice were randomly placed into cages for the remainder of their life before genotyping was performed. No other randomization was used while conducting experiments.

Statistical Analsysis-

Studies using animals were designed to minimize and control for confounding variables such as mouse strain, age, gender, time of day, the fed state of the animal and light cycling. Previous studies using inducible transgenic models have shown that treatment groups from 8 to 10 animals are sufficient to achieve statistical power when measuring RNA, protein, body weights, metabolic testing and blood metabolites.

UT Southwestern Medical Center has a site license for the use of GraphPad Prism software (version 6, GraphPad, Inc. San Diego CA). All data is depicted as mean plus and minus the SEM. All experiments presented have only two groups and unpaired students T-test was utilized for

statistical significance. All data compares Control mice to AiPKO mice under identical conditions. For all points * represents P-value<0.05, ** represents P-value<0.01, *** represents P-value<0.001 .

Metabolic testing

Oral glucose tolerance tests were performed as previously described (16). Briefly mice were fasted for 4 hours before a gastric gavage of glucose at 2.5 g/kg body weight. Glucose concentrations were measured using an oxidase-peroxidase assay (Sigma). Insulin levels were measured using commercial ELISAs from Millipore. Systemic insulin sensitivity was tested by performing an insulin tolerance test. Mice were fasted for 4 hours, and then injected with 1.0 IU/kg body weight of Humalin-R (Eli Lilly). Blood from the tail was measured for glucose content using Contour glucometer strips (Bayer) at 0, 15, 30, 60, 90 and 120 minutes. Pyruvate tolerance tests were performed by fasting mice for 4 hours, then injecting sodium pyruvate at ad dose of 2g/kg body weight. Blood glucose levels were measured from tail vein blood using Contour glucometer strips at -30, 0, 15, 30, 60 and 120 minutes post injection. 3 adrenergic receptor tests were performed as previously described (16). Briefly, mice were fasted for 4 hours, at which time *CL316,243* was administered intraperitoneally at 0.5 mg/kg body weight. Blood was collected at 0, 5, 15 and 30 minutes. Glycerol and NEFA measurements were determined using free glycerol reagent (Sigma) and free fatty acid quantification kits (Wako Diagnostics-NEFA-HR2) respectively.

Radioactive triolein chase experiments were performed as previously described (17). Briefly, unanesthetized mice were fasted for 4 hours, then subjected to an IV bolus tail vein injection of radioactive triolein which had been incorporated into 0.5% intralipid micelles. After 15 minutes, mice were sacrificed and tissues harvested. Using a chloroform:methanol lipid extraction protocol, oxidized triolein was separated from the lipid fraction and total incorporation, oxidized fraction and oxidized percent were calculated on a per gram tissue basis.

Amlexanox Treatment

To prepare amlexanox powder for gavage, 200mg of powder was resuspended in 11ml of 150mm NaOH. This solution was neutralized with 10ml of 1M pH7.4 tris-hcl. This solution was brought to 40ml making the final concentration of 5mg/ml. Mice were then treated daily by oral gavage at 50mg/kg body weight. Control gavage was prepared as the original 150mm NaOH then neutralized with tris-hcl and brought to volume, however Amlexanox was not added to this solution.

Quantitative RT-qPCR

RNA was isolated from tissues frozen on liquid nitrogen by homogenization in Trizol Reagent (Invitrogen) and RNA extraction using a RNAeasy RNA extraction kit (Qiagen). RNA was quantified using a Nanodrop Instrument. 800ng of RNA was used to transcribe cDNA using an Iscript kit (Invitrogen). The mRNA levels were calculated using the comparative threshold cycle (Ct) method.

Histology and immunofluorescence

Livers were excised and fixed overnight in 10% PBS buffered formalin and were thereafter stored in 50% ethanol. Tissues were sectioned (5um) rehydrated and stained using H+E

Adipocyte isolation

Adipocytes were isolated for confirmation of gene loss and determination of cell autonomous insulin sensitivity using a collagenase digestion similar to SVF isolation procedures (18). Briefly, freshly isolated adipose tissue was minced and subjected to 1 hour digestion at 37ºC in a PBS-collagenase buffer pH 7.4 (collagenase sigma (10mg/ml). This digest was centrifuged at 600xg for 5 minutes and the floating cells removed for RNA extraction.

References

1. Winzell MS, Ahren B. The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. Diabetes. 2004;53 Suppl 3:S215-9. Epub 2004/11/25. PubMed PMID: 15561913.
2. Fernandez-Real JM, Vayreda M, Richart C, Gutierrez C, Broch M, Vendrell J, et al. Circulating interleukin 6 levels, blood pressure, and insulin sensitivity in apparently healthy men and women. The Journal of clinical endocrinology and metabolism. 2001;86(3):1154-9. Epub 2001/03/10. doi: 10.1210/jcem.86.3.7305. PubMed PMID: 11238501.
3. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. Nature medicine. 2013;19(10):1252-63. Epub 2013/10/09. doi: 10.1038/nm.3361. PubMed PMID: 24100998.
4. Senn JJ, Klover PJ, Nowak IA, Mooney RA. Interleukin-6 induces cellular insulin resistance in hepatocytes. Diabetes. 2002;51(12):3391-9. Epub 2002/11/28. PubMed PMID: 12453891.
5. Nieto-Vazquez I, Fernandez-Veledo S, de Alvaro C, Lorenzo M. Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. Diabetes. 2008;57(12):3211-21. Epub 2008/09/18. doi: 10.2337/db07-1062. PubMed PMID: 18796617; PubMed Central PMCID: PMC2584126.
6. Franckhauser S, Elias I, Rotter Sopasakis V, Ferre T, Nagaev I, Andersson CX, et al. Overexpression of Il6 leads to hyperinsulinaemia, liver inflammation and reduced body weight in mice. Diabetologia. 2008;51(7):1306-16. Epub 2008/04/26. doi: 10.1007/s00125-008-0998-8. PubMed PMID: 18437347.
7. Vida M, Gavito AL, Pavon FJ, Bautista D, Serrano A, Suarez J, et al. Chronic administration of recombinant IL-6 upregulates lipogenic enzyme expression and aggravates high-fat-diet-induced steatosis in IL-6-deficient mice. Disease models & mechanisms. 2015;8(7):721-31. Epub 2015/06/03. doi: 10.1242/dmm.019166. PubMed PMID: 26035386; PubMed Central PMCID: PMC4486858.
8. Kraakman MJ, Kammoun HL, Allen TL, Deswaerte V, Henstridge DC, Estevez E, et al. Blocking IL-6 trans-signaling prevents high-fat diet-induced adipose tissue macrophage recruitment but does not improve insulin resistance. Cell metabolism. 2015;21(3):403-16. Epub 2015/03/05. doi: 10.1016/j.cmet.2015.02.006. PubMed PMID: 25738456.
9. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, et al. Interleukin-6-deficient mice develop mature-onset obesity. Nature medicine. 2002;8(1):75-9. Epub 2002/01/12. doi: 10.1038/nm0102-75. PubMed PMID: 11786910.
10. Mauer J, Chaurasia B, Goldau J, Vogt MC, Ruud J, Nguyen KD, et al. Signaling by IL-6 promotes alternative activation of macrophages to limit endotoxemia and obesity-associated resistance to insulin. Nature immunology. 2014;15(5):423-30. Epub 2014/04/01. doi: 10.1038/ni.2865. PubMed PMID: 24681566; PubMed Central PMCID: PMC4161471.
11. Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nature medicine. 2013;19(10):1338-44. Epub 2013/09/03. doi: 10.1038/nm.3324. PubMed PMID: 23995282; PubMed Central PMCID: PMC4075943.
12. Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, et al. An inhibitor of the protein kinases TBK1 and IKK-varepsilon improves obesity-related metabolic dysfunctions in mice. Nature medicine. 2013;19(3):313-21. Epub 2013/02/12. doi: 10.1038/nm.3082. PubMed PMID: 23396211; PubMed Central PMCID: PMC3594079.
13. Reilly SM, Ahmadian M, Zamarron BF, Chang L, Uhm M, Poirier B, et al. A subcutaneous adipose tissue-liver signalling axis controls hepatic gluconeogenesis. Nature communications. 2015;6:6047. Epub 2015/01/13. doi: 10.1038/ncomms7047. PubMed PMID: 25581158; PubMed Central PMCID: PMC4324568.

14. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. American journal of physiology Endocrinology and metabolism. 2001;280(5):E745-51. Epub 2001/04/05. PubMed PMID: 11287357.
15. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259(5091):87-91. Epub 1993/01/01. PubMed PMID: 7678183.
16. Kusminski CM, Holland WL, Sun K, Park J, Spurgin SB, Lin Y, et al. MitoNEET-driven alterations in adipocyte mitochondrial activity reveal a crucial adaptive process that preserves insulin sensitivity in obesity. Nature medicine. 2012;18(10):1539-49. Epub 2012/09/11. doi: 10.1038/nm.2899. PubMed PMID: 22961109; PubMed Central PMCID: PMC3745511.
17. Xia JY, Holland WL, Kusminski CM, Sun K, Sharma AX, Pearson MJ, et al. Targeted Induction of Ceramide Degradation Leads to Improved Systemic Metabolism and Reduced Hepatic Steatosis. Cell metabolism. 2015;22(2):266-78. Epub 2015/07/21. doi: 10.1016/j.cmet.2015.06.007. PubMed PMID: 26190650; PubMed Central PMCID: PMC4527941.
18. Gupta RK, Mepani RJ, Kleiner S, Lo JC, Khandekar MJ, Cohen P, et al. Zfp423 expression identifies committed preadipocytes and localizes to adipose endothelial and perivascular cells. Cell metabolism. 2012;15(2):230-9. Epub 2012/02/14. doi: 10.1016/j.cmet.2012.01.010. PubMed PMID: 22326224; PubMed Central PMCID: PMC3366493.

Conclusion

Adipose tissue is an incredibly powerful organ capable of a wide variety of functions. As a storage depot, adipose tissue provides a place to store potentially harmful nutrients in times of caloric excess; and subsequently is capable of redistributing these nutrients in times of nutrient paucity. The brown adipose depot is uniquely capable of keeping newborns and infants from dying due to cold exposure, as well as potentially providing the new frontier for combating insulin resistance and the sequelae of metabolic syndrome. Together these unique properties of adipose tissue make it indispensable for the survival and proliferation of mammals everywhere.

In chapter two of this work, I demonstrate the ability of properly functioning adipose tissue all by itself to prevent diabetes and other harmful pathologies. Using an inducible loss of function model to eliminate a gene which puts the brakes on insulin signaling in the adipocyte, I show how this storage vessel is capable of compensating for the metabolic insults brought on by high fat diet induced obesity. As PTEN regulates AKT activity, which in turn regulates Glut4 translocation to the plasma membrane, it is not surprising that this mouse has improved glucose tolerance when challenged during an oral glucose tolerance test. What is surprising is the immense capacity to which it can perform this function and for how long it can endure metabolic insults. After 5 months of high fat diet feeding, hepatic steatosis is essentially non-existent, and insulin stimulation is exquisitely increased in the liver by merely improving insulin sensitivity in the adipocyte. Moreover, this data demonstrates that inhibition of insulin resistance during high fat diet feeding can ameliorate the inflammation commonly seen in this state. This finding allows to better understand the “chicken and egg” relationship between insulin resistance and inflammation observed under these conditions.

This model still leaves questions however. It is interesting to note that enhanced insulin sensitivity in the adipose tissue does not enhance circulating adiponectin levels under chow fed conditions, since these

mice have equal circulating adiponectin levels. As adiponectin is known to be down-regulated under states of insulin resistance, it is interesting to note that the negative correlation with insulin resistance holds, while we are unable to build a positive correlation in a presumed state of maximal insulin sensitivity. So, under high fat fed conditions which induce insulin resistance, our more insulin-sensitive mice maintain higher adiponectin levels. Though in this scenario, we are unable to parse out whether we in fact see elevations in adiponectin secretion, or merely diminished decreases. This data suggests a positive regulatory role for insulin signaling, but is unable to solidify a direct causative relationship between these states.

In chapter 4 of this work, I demonstrate that IL-6 is not a mediator of metabolic dysfunction during high fat diet feeding. Furthermore, I identify the true nature of the drug Amlexanox and a potential therapeutic role of IL-6 signaling in adipose tissue *in vivo*. Inflammation in adipose tissue has taken center stage in the literature beginning in the mid 1990's. Since this time, much work has been centered on IL-6, as this cytokine is dramatically elevated in adipose tissue following high fat diet feeding in rodents. A great amount of work has demonstrated potential roles for IL-6 and its ability to inhibit insulin signaling. Herein, I show that during high fat diet feeding in rodents, IL-6 signaling via its primary receptor does not alter body weight, insulin sensitivity or glucose homeostasis. As this is the first inducible deletion of the IL-6 receptor in mature adipose tissue, this work stands to firmly rule out IL-6 as a mediator of the negative metabolic perturbations in adipose tissue following HFD challenge in rodents.

I go on to explore the functions of the new drug Amlexanox. This drug made a splash in the metabolism world, as it is already FDA approved for other conditions, and further showed tremendous abilities to combat weight loss and mediate improvements in metabolism following high fat diet challenge in rodents. In my work above, I show that Amlexanox indeed mediates weight loss and improvements in

glucose homeostasis; however these improvements are purely due to decreases in body mass and with rebound weight gain all improvements are lost. Importantly, my work goes on to show that other improvements in metabolism shown with Amlexanox are highly durable. Improvements in hepatic pyruvate tolerance, an indirect measure of gluconeogenesis, are stable months after the cessation of treatment, long after body weights have stabilized. My work shows that this response is due to enhanced β-adrenergic sensitivity in the adipose depots, a direct consequence of IL-6 signaling in these depots. With loss of this signaling using the KILRAT model, all improvements seen with Amlexanox treatment are abated and the mice show severe hepatic steatosis at more extended time points. Evidence for enhanced adrenergic sensitivity in depots beside the brown depot are supported by changes in gene expression and tissue weight in the subcutaneous depot, the most highly innervated of the peripheral adipose depots. Further, as we see a direct loss of improvements in pyruvate tolerance testing in the KILRAT models treated with Amlexanox, we confirm the role of brown adipose in protecting the liver from steatosis, as IL-6 in serum is equal between both of these models and therefore eliminates the direct action of IL-6 on the liver in initiating these improvements.

In summary, this work highlights the importance of adipose tissue in maintaining metabolic homeostasis. With high fat diet feeding, we see decreased insulin sensitivity as well as a less well appreciated decrease in β-adrenergic sensitivity. My data highlights the central role of both white and brown adipose to combat insulin resistance and the metabolic syndrome.