ADIPONECTIN AND TOLL-LIKE RECEPTOR 4: IMPORTANT ADIPOCYTE MODULATORS OF SYSTEMIC GLUCOSE AND LIPID METABOLISM

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DEDICATION

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ADIPONECTIN AND TOLL-LIKE RECEPTOR 4: IMPORTANT ADIPOCYTE MODULATORS OF SYSTEMIC GLUCOSE AND LIPID METABOLISM

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ADIPONECTIN AND TOLL-LIKE RECEPTOR 4: IMPORTANT ADIPOCYTE MODULATORS OF SYSTEMIC GLUCOSE AND LIPID METABOLISM

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As a global epidemic, the prevalence of obesity and its complications have increased rapidly over the past few decades. Obesity, characterized by excessive amount of body fat accumulation, is a strong predictor to various major health conditions such as type 2 diabetes and cardiovascular disease. Two hallmark features of an unhealthy hypertrophic adipose tissue are decreased adiponectin secretion and increased adipose tissue inflammation. Released almost exclusively from adipocytes, adiponectin exerts potent insulin sensitizing effects on peripheral tissues. Using a series of inducible mouse models, we identified an adipocyte-specific regulatory mechanism for adiponectin expression and release. In addition to the role in maintaining glucose homeostasis, adiponectin is also found to exert anti-fibrotic, anti-inflammatory and anti-apoptotic properties in numerous other cell types.

In the obese state, decreased adiponectin secretion contributes to increased adipose tissue inflammation. Toll-like receptor 4 is an important mediator of inflammatory response found abundantly on the cell surface of adipocytes. In this study, using an adipocyte- specific deletion, we demonstrated a dichotomous effect of Toll-like receptor 4 on adipose tissue functionality. Toll-like receptor 4 is essential for proper adipose tissue remodeling to promote healthy expansion during long term high-fat diet exposure. In contrast, toll-like receptor 4 can also be a mediator of insulin resistance during an acute challenge with saturated fatty acids.

In summary, my studies highlight a tight *in vivo* regulation of adiponectin secretion and demonstrate the role of adipocyte toll-like receptor 4 in modulating systemic glucose homeostasis during the development of obesity.

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LIST OF ABBREVIATIONS

ADNP – adiponectin
Alb- albumin
BAT- brown adipose tissue
Dox- doxycycline
FFAs- free fatty acids
FGF21- fibroblast growth factor 21
GFAP- glial fibrillary acidic protein
gWAT- gonadal white adipose tissue
het- heterozygous
HSCs- hepatic stellate cells
ITT- insulin tolerance test
KO- knockout
mWAT- mesenteric white adipose tissue
OGTT- oral glucose tolerance test
PTT- pyruvate tolerance test
rtTA- reverse tetracycline transactivator
S1P- sphingosine-1-phosphate
STZ- streptozotocin
svf- stromal vascular fraction
sWAT- subcutaneous white adipose tissue
TG- triglyceride
TLR4- Toll-like receptor 4
TRE- tetracycline response element
TZD- thiazolidinediones
WT- wildtype

CHAPTER ONE

INTRODUCTION

(The following section contain parts of a manuscript published in *Best Pract Res Clin Endocrinol Metab* 2014 PMID:24417945).

1:1 ADIPONECIN: MODULATOR OF GLUCOSE AND LIPID HOMEOSTATISS

Adiponectin has received considerable attention for its anti-diabetic actions. The adipokine exerts control of glucose and lipid homeostasis via critical effects within the liver, adipose, pancreas and other cell types. By stimulating adipogenesis, opposing inflammation, and influencing rates of lipid oxidation and lipolysis, adiponectin critically governs lipid spillover into non-adipose tissues.

Adipose tissue as an endocrine organ

Since the initial discovery in 1995, research on adiponectin has greatly shaped how we view adipose tissue. The multifaceted roles of adiponectin highlight the importance of adipose tissue as an endocrine organ (1,2). Adiponectin, first named Acrp30 (adipocyte complement related protein of 30 kD), is best defined functionally. Adiponectin is an adipocyte-specific secretory protein with potent insulin sensitizing, glucose lowering, and lipid catabolizing functions on peripheral tissues. Though adiponectin receptors (AdipoRs) were originally described as having tissue-specific distributions with differing isoforms restricted to skeletal muscle (AdipoR1) and liver (AdipoR2) (3), both receptors are relatively ubiquitous. Tracer studies with infrared dye-labeled adiponectin reveal prominent sites of adiponectin targeting in the liver, heart, and kidney, with the endocrine pancreas also being evident as a prominent binding site for full-length adiponectin when visualized with immune-fluorescent techniques (4,5). Furthermore, adiponectin is an excellent clinical marker for metabolic health. Consistent with the observed decreases in circulating adiponectin levels of obese individuals, numerous preclinical models have established roles for adiponectin in central and peripheral metabolic homeostasis (6-9).

Secretion of adiponectin

Adiponectin is secreted protein from adipocytes and circulates in a combination of 3 distinct complexes: trimers, low molecular weight multimers (LMW), and high-molecular weight oligomers (HMW). Circulating adiponectin levels inversely correlate with adiposity, as inflammatory mediators impair adiponectin production and release. A number of treatments with beneficial anti-diabetic effects are known to influence circulating levels of adiponectin, including thiazolidinediones (TZDs - PPARγ agonists), fibroblast growth factor 21(FGF21), anti-inflammatory compounds, and weight loss. Notably, adiponectin expression is essential for the effectiveness of glucose-lowering effects evoked by TZDs (10,11) or FGF21 (12,13). The HMW form of the protein is the best biomarker for clinical efficacy of TZDs (14) and is also markedly increased by FGF21 treatment (15). Additionally, diet and exercise, the first line of treatment for diabetes, can each elevate circulating adiponectin levels independently (16,17). Exercise may additionally facilitate adiponectin action by upregulating expression of adiponectin receptors (18,19). Collectively, the literature suggests that targeting adiponectin production or adiponectin signaling are attractive targets for therapeutic interventions for the prevention or treatment of obesity-related dysfunction in metabolism.

Physiological function of adiponectin

Adiponectin enhances hepatic insulin sensitivity and decreases hepatic glucose production.

Several laboratories have examined the effects of adiponectin on glucose and lipid metabolism. Berg et al. reported a two to five-fold elevation in circulating adiponectin levels can reduce plasma glucose levels in wildtype and diabetic mice (20). In addition, adiponectin knockout mice display glucose intolerance and severe hepatic, but not muscle insulin resistance upon high fat diet challenge (21). Injection of purified recombinant adiponectin during hyperinsulinemic-euglycemic clamp studies also leads to improved insulin action (5,22). The effects of adiponectin on *in vivo* glucose metabolism in the clamped state was attributed to a 65% reduction in the rate of glucose production in either WT or *ob/ob* backgrounds. Adiponectin did not affect the rates of glucose uptake, glycolysis, or glycogen synthesis. Instead, an acute increase in circulating adiponectin levels lowers hepatic glucose production

without affecting muscle glucose uptake. This is further evidenced by hepatic expression of the gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and glucose 6-phospharase (G6Pase). mRNA levels were reduced by more than 50% following adiponectin infusion, suggesting that a moderate rise in circulating adiponectin levels can inhibit both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production. Consistent with these rodent studies, fasting adiponectin levels strongly correlate with insulin sensitivity in humans (23). However, these glucose-lowering effects observed did not coincide with a rise in insulin.

Dogmatically, the mechanism by which adiponectin enhances insulin sensitivity was long thought to involve receptor-mediated activation of AMPK pathway to stimulate glucose utilization and fatty-acid oxidation (21,24,25). However, recent reports also reveal mechanisms independent of AMPK (26,27). Following an inducible reduction in LKB1/AMPK signaling in the liver, adiponectin still potently evoked improvements in glucose homeostasis. Receptor mediated ceramidase activity is a primary signaling mechanism by which adiponectin elicits broad spectrum of effects in the liver and elsewhere. Although ceramidase can promote AMPK activation, AMP Kinase signaling is not required for adiponectin to promote ceramidase activation or ceramide-lowering effects (27). Miller and colleagues convincingly demonstrated that, in the absence of LKB1/AMPK signaling, adiponectin can still reduce gluconeogenic gene expression and facilitate insulin-induced suppression of hepatic glucose efflux during hyperinsulinemic-euglycemic clamps (28). Targeted inhibition of *de novo* ceramide synthesis can normalize hepatic ceramide and restore insulin's ability to suppress hepatic glucose output in a number of animal models. The kinetics of adiponectin-mediated improvements in hepatic insulin action overlap with the time required for adiponectin to normalize ceramide levels in the livers of *ob/ob* mice (5). Although ceramides have additionally been shown to accumulate in the livers of obese insulin resistant humans (29), the role of ceramides in hepatic insulin resistance in humans remains under scrutiny (30), but is likely to have a similarly negative role on insulin signaling as in rodents.

Enhancements in circulating adiponectin also strongly correlate with clinical efficacy of insulin sensitizers. Most notably, PPARγ agonists are strongly linked with adiponectin. In

preclinical models, adiponectin is essential for TZDs to promote efficient improvements in glycemia, particularly at low doses (10,11). In humans, TZD-induced improvements in gluconeogenesis strongly correlate with increases in circulating adiponectin (31). The HMW form of adiponectin is the best predictor for improvements in glucose homeostasis evoked by TZDs (32). This circulating form is also a better correlate for insulin sensitivity in general than total adiponectin (33-35). These correlations also extend for other effects of TZDs, particularly TZD induced decreases in hepatic steatosis, fibrosis, and inflammation seen in NASH patients (36).

Adiponectin lowers ceramide accumulation and prevents apoptosis.

Ceramides are a family of lipids composed of sphingosine backbone and a fatty acid. *De novo* ceramide synthesis starts with serine and palmitoyl Co-A to form an 18-carbon backbone. Following by multiple enzymatic reactions, ceramide is formed. Ceramides can inhibit insulin action via diminished signaling of Akt, a central kinase involved in insulin signal transduction (39). As such, high levels of intracellular ceramides are associated with reduced nutrient uptake, decreased insulin sensitivity, and increased apoptosis. The deacylation of ceramide, characterized by the release of a sphingosine and a free fatty acid, is carried out by an enzyme called ceramidase. Once converted from ceramide, sphingosine can be phosphorylated by sphingosine kinase to form sphingosine-1-phosphate (S1P) (27,39). S1P is known to exhibit opposite effects to ceramide, in the sense that it can promote cell survival, improve insulin sensitivity, and reduce inflammation. Therefore, the relative ratios of ceramide and S1P are crucial for survival and insulin sensitivity of the cell. Hence, the modulation of ceramide metabolism is essential in maintaining metabolic homeostasis. Because of the similar beneficial metabolic functions between adiponectin and S1P, addressing the relationship between the two molecules has received recent attention.

The regulation of ceramide metabolism is tightly associated with lipid intake, increased by inflammatory mediators, and decreased by adiponectin (40). Using genetic gain or loss of adiponectin receptors in cell culture experiments further clarified the role of adiponectin in inducing a ceramidase activity mediated via its canonical receptors. This is supported through research showing a heterologous system connecting this class of receptors with ceramidase

activity (41,42). These receptors convey ceramidase activity that can be further enhanced by adiponectin, which results in simultaneous decreases in ceramides and increases in S1P. Collectively, these data suggest activation of AdipoR1 and R2 induces up-regulation of ceramidase activity and is ultimately favoring the production of S1P (27,39). The resulting sphingosine and S1P produced in this process may be sufficient to activate PPARγ and AMPK, the downstream mediators of adiponectin signaling. S1P addition is sufficient to induce AMPK phosphorylation (5), and sphingosine has been reported as a ligand for PPARs (43). In the context of lipid oxidation, ceramide may contribute to impairments in lipid oxidation, as it promotes de-activation of AMPK via activation of protein phosphatase 2a (PP2A) (44-46).

This local and systemic increase in S1P likely conveys the anti-apoptotic effects of adiponectin, as S1P is sufficient to recapitulate the protective effects of adiponectin in cardiomyocytes and pancreatic beta cells. In other words, adiponectin lowers ceramide content and prevents apoptosis through adiponectin-receptor mediated increased ceramidase activity, i.e. driving the ceramide: S1P rheostat toward S1P mediated survival and proliferation. Tissues are constantly in equilibrium between death, survival and proliferation/renewal. Adiponectin is critical for cell survival and self-renewal for several tissues (5,47)

Adiponectin promotes the maintenance of β cell mass

In a normal pancreas, a subpopulation of endocrine cells known as β cells constitute the predominant type of cells in clusters called islets of Langerhans. These cells are responsible for the secretion of the peptide hormone insulin, which promotes the uptake of carbohydrates and nutrients in skeletal muscle and fat, while suppressing glucose efflux from the liver and lipolysis from adipose. While the majority of metabolic studies evaluating adiponectin effects on glucose homeostasis have examined insulin resistance, several lines of evidence suggest that adiponectin may additionally promote β cell survival. Compounds such as sulfatides and TZDs known to prevent diabetes in non-obese diabetic (NOD) mice or genetically predisposed humans increase circulating levels of adiponectin (48,49). Adiponectin prevents lipid, ceramide or cytokine (interleukin-1β+interferon-γ)-induced apoptosis in cultured INS-1 β cells (5,50). A 3-4-fold overexpression of full-length adiponectin maintains β cell mass and glucose homeostasis in *ob/ob* mice and a model of type 1 diabetes (5,51). Adiponectin null mice are

more susceptible to caspase-8-induced β–cell apoptosis, and cells from adiponectin receptor deficient animals are highly prone to lipoapoptosis (5). Additionally, in terms of β–cell function, adiponectin maintains glucose stimulated insulin secretion when the β–cell is challenged with lipid or cytokine (50), but it does not consistently affect insulin secretion in the absence of such insults (50,52-54).

The cytoprotective effects of adiponectin are highly relevant to the β cell. Sphingolipids, such as ceramides and glucosylceramides, are an important class of bioactive lipids. The levels of these lipids change as a function of adipose tissue mass and functionality, and are partially driven by cellular availability of palmitoyl-CoA. Aberrant accumulation of ceramide has been strongly implicated in lipotoxic β cell failure by our colleague Roger Unger (55-58). Adiponectin targets the endocrine pancreas (but not the exocrine pancreas) and protects β–cells from apoptosis. In an acute model of β cell decompensation, only modest hyperglycemia was observed. In contrast, after the same treatment conditions, mice that overexpress adiponectin (3-4 fold increase in circulating levels) are completely euglycemic and maintain a larger islet area. Contrary, β cell decompensation resulted in enhanced loss of pancreatic insulin content and led to smaller islets in adiponectin null mice (5). Combined, these results highlight the potent cytoprotective effects that adiponectin exerts on β–cells *in vivo*.

Adiponectin opposes steatosis.

Dietary lipids are metabolized primarily in the intestines, where they are broken down, rebuilt into triglycerides (TGs), and then repackaged with cholesterol and proteins into chylomicrons. Chylomicrons are intestinally secreted lipoprotein particles that function to transport exogenous lipids through the bloodstream primarily to the liver where lipids can be repackaged into lipoprotein particles. As lipoproteins reach target tissues, TGs are unloaded by lipoprotein lipase and undergo subsequent hydrolysis to release glycerol and free fatty acids (FFAs), which then enter the cell and function as an energy source, or a building block for membrane lipid synthesis. Excess lipid can additionally be stored in target tissues by re-esterifying FFAs into TGs. Though adipose is the primary tissue for lipid storage, during

nutrient overload, excess energy intake can also be stored in liver in the form of TGs. Upon energy demand, the stored TGs can be broken down into FFAs through lipolysis (59).

Preclinical models indicate roles for adiponectin in the maintenance of hepatic lipid metabolism. Adiponectin null mice develop fibrotic steatohepatitis and adenomas when maintained on high fat diets for 48 weeks (60), but not by shorter-term diet administration (61,62). Genetic ablation of adiponectin in *ob/ob* mice further exacerbates hepatic triglyceride accumulation (12). Conversely, adiponectin overexpression prevents accumulation of TG or the deleterious lipid metabolites diacylglycerol or ceramides (51,63). Similar effects are seen in other transgenic mice which develop hyperadiponectinemia secondary to changes in adipose mitochondrial function, as they are also refractory to TG, diacylglycerol or ceramide over-accumulation (64). Administration of recombinant adiponectin in rodents results in beneficial effects on lipid metabolism, such as enhancing lipid clearance and increasing fatty acid oxidation in muscle and liver (65,66). Several groups have also demonstrated that circulating adiponectin concentrations decrease during chronic ethanol exposure in rodents (67-69). Adenoviral adiponectin overexpression can attenuate hepatomegaly, steatosis and liver injury in mice exposed to chronic ethanol added into their high fat diet. This high fat diet / ethanol treatment lowers plasma adiponectin within 3 weeks as steatosis develops (68).

Adiponectin is anti-steatotic by decreasing free FFA influx into the liver and increasing FFA oxidation and mitochondrial biogenesis. Though not consistently seen in either human or animal studies (74), some reports indicate a downregulation of adiponectin receptor transcription in patients with fatty liver (75,76). Decreased expression of AdipoR2 has also been observed in murine models of hepatic steatosis (77). Conversely, the direct manipulation of adiponectin receptor expression demonstrates a potential causal relationship between adiponectin signaling and steatosis (78). Adenoviral-mediated overexpression of either adiponectin receptor is sufficient to stimulate lipid oxidation and diminish hepatic TG content (79). By contrast, mice lacking both isoforms of adiponectin receptor display enhanced hepatic TG accumulation. Combined, the combinatory effects of low plasma adiponectin and low AdipoR2 in liver may each contribute to the development of hepatic steatosis.

Adiponectin is anti-fibrotic

Fibrogenesis is a complex wound-healing response involving multiple aspects, such as nuclear receptor activation, inflammatory cytokines, and growth factors. Prolonged fibrogenesis can ultimately lead to fibrosis and cirrhosis. Fibrosis, giving rise to scarring tissue, is characterized by an accumulation of fibrillar collagen (collagen I and III) in the extracellular matrix (ECM). It has been shown that activation of hepatic stellate cells (HSCs) and Kupffer cells modulate the hepatic fibrogenic process in injured liver tissue (80). Not all, but most, of the fibrotic cells are derived from activated HSCs (81,82). Upon liver injury, quiescent HSCs lose partial structural integrity and their droplet function as they switch on collagen production and become more proliferative. Activated HSCs are fibrogenic and can feedback to activate more HSCs via amplifying inflammatory responses (83-85).

An additional discovery supporting adiponectin's anti-fibrotic potential is that quiescent HSC display adipocyte-like properties, and they are able to secrete adiponectin. However, upon activation, instead of adiponectin, HSCs increase leptin production and reduce adiponectin production. Based on these findings, previous studies have demonstrated the potential of using adiponectin for regression of fibrogenesis (70,81,86). In collaboration with our laboratory, Shafiei et.al reported that adiponectin overexpressing transgenic mice receiving thioacetamide were resistant to fibrosis, compared to controls. In contrast, adiponectin knockout animals developed severe fibrosis (86).

Adiponectin is anti-inflammatory

Lipotoxicity describes the condition of cellular dysfunction or apoptosis as a consequence of unfavorable lipid deposition in tissues other than adipose tissue (87,88). When the net storage of TG exceeds its limits, the accumulated lipid will impair regular cell function and promote apoptosis. Along with the loss of functional adipocytes and hepatocytes, apoptosis, inflammatory cytokines and various additional stress signals are also released; thus creating a toxic environment for the surrounding cells. This toxicity can trigger cell defense and repair mechanisms, which include fibrogenesis and apoptosis (89,90).

Toll-like receptor 4 (TLR4) can be activated as a consequence of aberrant lipid homeostasis. Recent publications have indicated saturated fatty acid can indirectly activate

TLR4 signaling initiate inflammatory response and production of ceramide biosynthetic enzymes (91-96). Direct and indirect activation of TLR4 receptor activates the downstream NF-κB signaling pathway. This activation process will lead to increased pro-inflammatory cytokine production and up-regulation of ceramide production (91). Ceramide itself has also been implicated as an inducer of inflammatory signaling via activation of IKKβ and Jun N-terminal kinase (JNK) (97,98). In acute inflammation, pro-inflammatory cytokines have a short half-life, therefore limiting the duration of the inflammatory response. To tightly control the inflammatory response, anti-inflammatory cytokines can counter immune activation and attenuate the activation of pro-inflammatory cytokines. This regulation is further accompanied by macrophage-mediated removal of the dead cells, thereby terminating the source of pro-inflammatory signals (99).

Using macrophage-like cell lines, Hung et al. reported normalized TLR-4 mediated signaling with globular adiponectin (100). A similar research using primary Kupffer cells has also demonstrated the LPS desensitizing, anti-inflammatory effect of adiponectin (101). However, both of these results are likely due to a desensitization event due to LPS contamination of the globular adiponectin preparation. Other data is more directly supportive of an ant-inflammatory role of adiponectin by attenuating activation of pro-inflammatory response, NF-κB signaling, and most importantly, by decreasing ceramide accumulation in tissues (27,102).

Adiponectin facilitates lipid sequestering by adipose

Healthy adipose tissue acts like a sponge to absorb extra nutrients and store them in inert TG droplets for subsequent release during energy-deplete conditions. Adiponectin promotes this metabolic flexibility by facilitating adipose expansion, preventing adipose inflammation and fibrosis, and maintaining adipose tissue health (103).

Adiponectin can enhance healthy adipose tissue expansion. Our lab has demonstrated roles for adiponectin in adipose expansion in two distinct animal models. Conversely, adiponectin knockout mice are more sensitive to lipolysis (104). Kim et al. reported that aP2-driven (adipose restricted) adiponectin overproduction in leptin deficient *ob/ob* mice results in pronounced expansion of healthy adipose tissue, consisting of smaller adipocytes and reduced

macrophage infiltration (51). Despite being far more obese than their *ob/ob* littermates, these adiponectin transgenic-*ob/ob* mice display normal glucose tolerance, decreased hepatic steatosis, and normal islet architecture. Marked lowering of serum TGs, enhanced triglyceride clearance, improved HDL:LDL profiles, and lower FFA levels were all noted. These improvements in lipid profiles appear largely driven by enhanced lipoprotein lipase activity in adipose and subsequent increases in adipose lipid uptake. These fat pads were highly effective at limiting lipid spillover to other tissues and diminished formation of lipid metabolites in liver. Increases in whole-body energy expenditure were also evident with enhanced mitochondrial proliferation in white adipose (51).

Similar improvements in glucose homeostasis have also been noted in mice with abundant adiponectin occurring secondary to altered expression of a mitochondrial outer-membrane protein termed MitoNEET (64). These aP2-driven MitoNEET-overexpressing mice offer remarkable similarities to adiponectin transgenic mice, though they have impaired lipid oxidation due to overexpression of this mitochondrial protein. Despite record-setting obesity, they are completely glucose tolerant with remarkable hepatic insulin sensitivity, even on a leptin deficient background. Like adiponectin transgenic mice, MitoNEET transgenics have enhanced adipose LPL activity and additionally show enhanced expression of fatty acid transport proteins. These offer a remarkable capacity to clear triglycerides from the bloodstream and store them in adipocytes, without developing adipose fibrosis or inflammation. This lead to decreased accumulation of TG and lipid metabolites in the liver, and maintained islet architecture. Thus, adiponectin overexpression or hypersecretion in response to metabolic perturbations profoundly enhances adipose tissue expansion and function, sparing non-adipocytes from ectopic lipid accumulation.

Summary

Adiponectin can exert pleiotropic effects on various tissues under many metabolic conditions. Work done in the past decade has uncovered part of the regulation and the mechanism of adiponectin action. However, despite the intensive characterization of the physiological function of adiponectin, there are still gaps to fill with regards to the molecular mechanism of its biogenesis and mechanism of action. To name a few: the physiological

function of the different adiponectin complexes, the effects of intracellular adiponectin, and the local effects of adiponectin within specific tissues. Addressing these unanswered questions can further strengthen the potential of using adiponectin as therapeutic options in multiple metabolic conditions in the future.

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1:2 TOLL-LIKE RECEPTOR 4 LINKING OBESITY-ASSOCIATED INFLAMMATION WITH INSULIN RESISTANCE

Toll-like receptor 4 (TLR4) plays a critical role in the innate immune system by activating pro-inflammatory signaling pathways in response to lipopolysaccharide (LPS) present on microbial pathogens. Inflammatory pathways are activated in the tissues of obese animals and humans. TLR4 gained considerable attention in the context of obesity-associated inflammation since 2006. Several groups have demonstrated that TLR4 can initiate inflammatory cascades in response to saturated fatty acids, similar to LPS activation; suggesting that TLR4 may be a critical player in the regulation of energy balance and insulin resistance in response to changes in the nutritional environment.

Activation of toll-like receptor 4

The Toll-like receptor (TLR) family is part of the group of pattern recognition receptors (PRRs) which function to recognize molecules shared among pathogens to initiate host defense innate immune responses (1). TLR4 is the best-characterized TLR; TLR4 can recognize and bind to LPS present on the cell wall of gram-negative bacteria. Upon binding, the adaptor protein myeloid differentiation factor (Myd88) is recruited to the receptor. This interaction then triggers the downstream signaling cascade, leading to activation of the NF-κB pathway, which subsequently activates transcription of several pro-inflammatory genes. Recent data has demonstrated an alternative activation of the TLR4 signaling by saturated fatty acids. One possible mechanism accounting for the FFA activation is through adaptor protein fetuin-A (2) Regardless of the ligand, activated TLR4 signaling can initiate series of signaling cascades that ultimately result in pro-inflammatory cytokine production, such as TNF-α and TGF-β.

Inflammation and metabolic dysregulation

TLR4 in the development of liver fibrosis

Activation of TLR4 signaling can stimulate hepatic stellate cells and regulate cell survival and apoptosis (3-7). In addition to LPS activation, the TLR4 signaling pathway can

also be activated in response to ER stress induced by FFAs and the resulting ectopic lipid storage in hepatocytes. This activation can contribute to both the induction of unfolded protein response (UPR) and the upregulation of an inflammatory response (8,9). TNF-α and TGF-β, cytokines downstream of TLR4, can signal back to active HSCs to enhance collagen production, fibrogenesis, and hepatocyte apoptosis (3,4,10).

TLR4 in obesity-associated inflammation

Obesity is associated with increased local adipose inflammation and macrophage infiltration. Direct and indirect activation of TLR4 activates the downstream NF-κB signaling pathway. This activation process will lead to increased pro-inflammatory cytokine production and up-regulation of ceramide production (11). In acute inflammation, pro-inflammatory cytokines have a short half-life, therefore limiting the duration of the inflammatory response. To tightly control the inflammatory response, anti-inflammatory cytokines can counter immune activation and attenuate the activation of pro-inflammatory cytokines. This regulation is further accompanied by macrophage-mediated removal of the dead cells, thereby terminating the source of pro-inflammatory signals (12).

During adipose tissue expansion, various acute phase reactants and mediators of inflammation, such as TNF-α, IL1-β and IL-6, can be secreted from adipocytes (13,14). Increased production of inflammatory cytokines from adipocytes and macrophages both contribute to the increased local adipose inflammation. Excess nutrients can lead to further adipose tissue expansion and ectopic lipid storage (15,16). This unhealthy adipose tissue expansion is associated with necrotic adipose tissue, decreased adiponectin secretion and increased pro-inflammatory cytokine secretion (13). The inflamed adipose tissue then contributes to the development of systemic inflammation. Obesity-associated adipose tissue inflammation and systemic inflammation, together with decreased circulating adiponectin levels will lead to further activation of the TLR4 signaling pathway and decreased adiponectin receptor binding until the signal resolved (17,18).

In chronic inflammation, the balance between pro- and anti-inflammatory cytokines and macrophages is dysregulated. Instead of undergoing phagocytosis, recruited macrophages are turned into a secondary source for the production of pro-inflammatory cytokines. The

consequence of this dysregulation is the formation of a feed-forward loop starting with unresolved inflammatory response, following by the accumulation of dead cells and macrophage recruitment, which in turn leads to further pro-inflammatory cytokine production through activation of TLR4.

TLR4 in the development of obesity-induced insulin resistance

A number of studies have shown the connection between the innate inflammatory response, type 2 diabetes, and insulin resistance (11,17-21). Activation of TLR4 signaling and the subsequent release of inflammatory cytokines through regulation of several important transcription factors is critical in connecting obesity-associated inflammation and insulin resistance (19). Studies using globally TLR4-deficient mice revealed attenuated diet-induced inflammation and insulin resistance (22-24). However, the specific cell type(s) mediating these effects is/are unclear, since TLR4 is widely expressed throughout the body. One possible mechanism linking TLR4 activation with insulin resistance is through ceramide biosynthesis. The regulation of ceramide metabolism is tightly associated with lipid intake and closely modulated by inflammatory responses and adiponectin. Activation of TLR4 by FFAs can stimulate ceramide production and accumulation. The accumulation of intracellular ceramide then leads to inhibition of Akt signaling, a key downstream mediator of the insulin signaling pathway, and also impairs insulin sensitivity of the cell (17,25,26). In other words, there is a crosstalk between innate immunity and insulin sensitivity through ceramide metabolism.

Summary

In mild obesity, adipose tissue is expanding. The healthy expansion of the adipocyte is associated with a slight increase in the inflammatory response and the recruitment of M1 macrophages. At this stage, the adipocyte is experiencing mild metabolic dysfunction. However, its ability to secret adiponectin is not affected, and potentially even slightly increased (12,13,21,27). The adipocyte is still able to maintain a balance between pro- and anti-inflammatory cytokine production, and attempts to resolve nutritional stress. Taking a step further, an over-expanded, unhealthy adipocyte is very likely to be metabolically fully dysfunctional. Histology sections show the presence of crown-like structures, reflecting

adipocyte necrosis and widespread macrophage infiltration under these conditions (13,21,27). In addition, the over-expanded adipocyte has lost its ability to secret a sufficient amount of adiponectin. Necrotic adipocytes, together with the massively enlarged remaining adipocytes, then serve as sites of pro-inflammatory cytokine production (28). The inability of the unhealthy adipocyte to resolve existing inflammatory responses closely resembles what is observed in the context of chronic inflammation. Two direct consequences of such unhealthy expansion are: decreased adiponectin secretion and increased net production of pro-inflammatory cytokines. Systemically, the two events lead to the development of obesity-associated insulin resistance in other tissues, such as the liver, via decreasing receptor-mediated ceramidase activity and increasing intracellular ceramide production through the TLR4-NF-κB axis (17,26).

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CHAPTER TWO

ECTOPIC ADIPONECTIN OVEREXPRESSION

(The following section is part of a manuscript prepared for submission)

2:1 ADIPOCYTE-SPECIFIC INHIBITION OF ECTOPIC ADIPONECTIN EXPRESSION *IN VIVO*

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Abstract

The pleiotropic actions of adiponectin in the context of metabolism make it a very useful biomarker as well as a direct mediator of improvements in the diabetic and obese state. We generated a transgenic animal expressing full-length adiponectin under the control of a tetracycline responsive element (TRE)-driven promoter. Within 48 hours of induction, we were able to observe high levels of plasma adiponectin when the transgene is expressed in the hepatocyte (TRE-apn/Alb-rtTA). The same transgene was also bred to express exclusively in adipocytes (TRE-apn/WT). However, ectopic adiponectin induction from adipocytes resulted in a *reduction* in plasma adiponectin levels. The observed regulatory mechanism on adiponectin expression is independent of the amount of endogenous adiponectin. In contrast to the situation *in vivo,* cultured differentiated adipocytes using the stromal vascular fraction (SVF) of the subcutaneous white adipose tissue (sWAT) from the adipocyte overexpression animals in adiponectin knockout background (TRE-apn/KO) demonstrate that these cells produce and secrete ectopic adiponectin upon induction without any issues. *In vivo* experiments suggest that insulin signaling and adipose tissue innervation contribute at least in part to the suppression of ectopic adiponectin production *in vivo*. These results expand the current understanding of adiponectin production *in vivo* and highlight the importance of the adipocyte-specific post-transcriptional regulation of adiponectin release.

Introduction

Obesity and related metabolic diseases have become a major threat to global health (1). The adverse effects of excess adipose tissue begin when the energy intake exceeds the remodeling capacity of adipose tissue, subsequently leading to adipocyte hypertrophy (2). A hypertrophic dysfunctional adipose tissue is rapidly undergoing increased ER stress, hypoxia, macrophage infiltration and enhanced lipolysis due to insulin resistance. This unhealthy adipose tissue can then affect other peripheral tissues through the release of adipokines and excessive amount of free fatty acids and other lipid derivatives, such as ceramides. Impaired insulin signaling and ectopic lipid deposition are both among the most obvious consequences of a dysfunctional adipose tissue (3).

A hallmark of unhealthy adipose tissue is the reduction of adiponectin secretion. While most adipokine levels increase with obesity, plasma adiponectin levels tend to decrease in obesity. Adiponectin is a member of the family of complement factor C1q-like proteins. Adiponectin (at the time referred to as Acrp30) was first characterized in 1995 as a secretory protein made exclusively in adipocytes (4). Since then, many groups have reported on its role in energy homeostasis and obesity in rodents, non-human primates and humans (5,6). The adipose tissue-specific expression of adiponectin and its profound impact on systemic energy homeostasis highlight the importance of adipose tissue as an endocrine organ. In the complex crosstalk between the adipocyte and other cell types, adiponectin has an established place as an important anti-lipotoxic agent amongst a number of other adipokines.

Thus far, adiponectin is best known for its insulin sensitizing, glucose lowering and lipid catabolizing functions on peripheral tissues (7). Through the past 15 years, clinical studies have revealed positive correlations between plasma adiponectin levels and numerous cell-protective effects, such as anti-inflammatory, anti-apoptotic and anti-fibrotic actions in various metabolically challenged settings (8-11). In addition, tracer studies with infrared-labeled adiponectin uncover prominent sites of adiponectin targeting in the liver, heart, the pancreatic β cells and kidney (12). Consistent with the clinical observations, pre-clinical models have more directly established the beneficial and protective roles of adiponectin in central and peripheral metabolic homeostasis (13,14).

Keeping in mind the physiological functions of adiponectin and the negative correlations in between plasma adiponectin levels and the development of obesity and related metabolic diseases, we set out to better understand how we can increase the production and release of adiponectin from the adipocyte. To address this question, we generated a transgenic mouse strain that allows us to overexpress adiponectin in a target cell-specific and doxycycline (Dox)-inducible form (referred to a *tet*-responsive element driven adiponectin, or "TRE-apn"). By utilizing this animal with differential target cell-specific activators, we uncovered a cellular regulatory pathway *in vivo* that seems to be unique to adipocytes. This regulation was lost *in vitro* upon using differentiated adipocytes cultured from the stromal vascular fraction (SVF) of the transgenic animals. In addition, our studies also offer insights into the local protective effect of adiponectin overexpression in liver. Despite the adipocyte specific regulation observed *in vivo*, our attempts for overexpression were also performed in the adiponectin knockout (KO) background and suggested that even low levels of adiponectin are sufficient to improved glucose metabolism during chronic high fat diet (HFD) feeding. Collectively, our studies demonstrate adiponectin's pleiotropic actions in both adipose and peripheral tissues and highlight as well the unique features of the regulation of adiponectin secretion *in vivo*.

Results

Ectopic overexpression of adiponectin from adipocytes results in reduced plasma adiponectin levels

We generated a transgenic animal expressing full-length adiponectin under the control of TRE promoter. Upon breeding this strain with a transgenic animal carrying a construct containing the adiponectin promoter driven "*tet-on*" transcription factor rtTA, the progeny carrying both the TRE and the rtTA transgenes can inducibly express adiponectin exclusively from the adipose tissue upon induction with doxycycline ("Dox"). We refer to these mice as TRE-apn/WT (**Fig. 1a**). Upon seven days of Dox exposure in the food, we observed a 3-fold increase in total adiponectin (ADPN) mRNA levels, attributed to the induction of the exogenous adiponectin (TRE-apn) mRNA in the sWAT (**Fig. 1b**). Surprisingly, we did not observe any accumulation of protein, neither within the sWAT nor in circulation. Similar observations were made for other fat pads (not shown). In fact, there was even a reduction in plasma adiponectin in TRE-apn/WT when compared with wildtype littermates (**Fig. 1c**). On the basis of these observations, we hypothesized that endogenous adiponectin release is subject to a feedback loop to prevent adiponectin overexpression.

Adiponectin overexpression from hepatocytes, in contrast to adipocytes, gives rise to high circulating adiponectin levels

We wanted to test if this feedback mechanism is unique to adipocytes or whether other cell types would behave in a similar way. The same TRE-apn transgenic animal was bred with a hepatocyte-specific rtTA mouse to generate a hepatocyte-specific overexpression model (TRE-apn/Alb-rtTA) (**Fig. 2a**). After seven days of Dox induction, we were able to detect adiponectin mRNA in the liver in response to transgene expression. Adiponectin overexpression from the liver did not affect the endogenous adiponectin mRNA expression in adipose tissues (**Fig. 2b-c**). In addition, we were able to observe very high levels of circulating adiponectin from the TRE-apn/Alb-rtTA mice. This liver-derived adiponectin did not affect the intracellular adiponectin level in the sWAT, nor did it accumulate at disproportionately high levels within the liver (**Fig. 2d**). This suggests that exogenous circulating adiponectin, even at very high levels, does not exert feedback on adipocytes to suppress adiponectin production in the same way adipocyte-specific overexpression does. Since we can achieve very high-level

overexpression of liver-derived adiponectin in circulation, yet liver tissue does not stand out as accumulating high levels intracellularly, the high serum concentration may be the result of a decreased clearance. Indeed, we had to dilute plasma from the liver-derived adiponectin 600 times to achieve similar plasma adiponectin concentrations as wildtype animals, suggesting that we achieve low mg/ml levels of adiponectin in circulation as opposed to μg/ml concentrations of endogenous adiponectin.

To determine the half-life of this liver-derived adiponectin, we tail vein-injected serum collected from wildtype animals and TRE-apn/Alb-rtTA mice into adiponectin KO recipients and monitored the clearance. During a 24-hour time course, we found that the liver-derived adiponectin has a significantly longer half-life than endogenous adiponectin, with approximately 150 minutes compared to the 30- 45 minutes seen for the adipocyte-derived protein (**Figs. 2e-f**).

Aside from the differences in half-life, the liver derived adiponectin seems to be functional. TRE-apn/Alb-rtTA animals were more resistant to HFD-induced weight gain during an 8-week high fat diet (HFD) challenge (**Suppl. Fig. 1a**). The TRE-apn/Alb-rtTA mice were more glucose tolerant during an oral glucose tolerance test (OGTT), and that improved glucose tolerance was not the result of enhanced insulin secretion during the OGTT (**Suppl. Fig. 1b**). However, to test whether we can observe enhanced responses to insulin under these conditions, an insulin tolerance test (ITT) was performed. However, this crude test did not reveal any differences in insulin sensitivity (**Suppl. Fig. 1c**). In contrast, decreased hepatic glucose production was observed during a pyruvate tolerance test (PTT) (**Suppl. Fig. 1d**). Also, over the course of an oral lipid gavage, we noticed significantly improved lipid clearance in the TRE-apn/Alb-rtTA mice (**Suppl. Fig. 1e**). So we see all the hallmarks of adiponectin-mediated improvements in glycemic and lipid control, though we do not know whether this activity would be comparable to adipocyte-derived adiponectin on a molar basis, since we overexpress adiponectin to such high levels in the liver transgenic animals. Furthermore, it is apparent that hepatocyte-derived and endogenous adipocyte-derived adiponectin are in fact differentially post-translationally modified, evidenced by the shift in molecular weight apparent

in western blotting, which may in fact explain the differential clearance rates and may affect bioactivity as well (**Fig. 2e**).

Interestingly though, local hepatocyte-specific overexpression of adiponectin did not protect the livers from carbon tetrachloride-induced liver fibrosis, in fact, it made the mice even more susceptible (**Suppl. Figs. 1f-g**). In contrast to these observations, local overexpression of adiponectin from hepatic stellate cells did, however, prevent the development of fibrosis under the same conditions (*Tao and Scherer, manuscript in preparation*).

Adipocyte-specific inhibition of ectopic adiponectin overexpression *in vivo*

To further examine whether the adipocyte simply "caps" its adiponectin production at systemically determined levels, we decided to test the ability of the adipocyte to produce ectopic adiponectin in the absence of endogenous adiponectin. The absence of endogenous adiponectin should allow the transgenic adiponectin to be expressed at least at levels comparable to wildtype. To test this, we bred the TRE-apn/WT animals into a whole body adiponectin KO background (TRE-apn/KO). Surprisingly, even in the absence of endogenous adiponectin, we failed to observe any adiponectin production at the protein level despite the induction of adiponectin at mRNA level (**Fig. 3a-b**). However, even at full induction, TRE-apn/KO only achieved about 2% of endogenous adiponectin mRNA levels seen in a WT fat pad (**Fig. 3c**).

To our surprise, we encountered an entirely different situation when we examined cells from these mice *in vitro*. We harvested the stromal vascular fraction from TRE-apn/KO fat pads and differentiated these cells into adipocytes. Culturing and differentiating the SVF cells from sWAT revealed that these cells are fully capable to produce and secrete ectopic adiponectin upon induction to an extent comparable to what differentiated SVF cells from a WT fat pad can do (**Fig. 3d**). Addition of proteasomal and lysosomal inhibitors did not seem to affect the adiponectin content, neither in the collected medium nor within the cells. The adiponectin mRNA levels, attributed solely to the transgene in the TRE-apn/KO, were about 4-fold higher than WT under the same conditions *in vitro* (**Fig. 3e**). Therefore, the adipocyte-specific regulation of adiponectin expression is unique to the *in vivo* setting, and does not apply to *in vitro* conditions.

Despite the extremely low adiponectin protein levels *in vivo* in the TRE-apn/KO mice, a small fraction of adiponectin escapes from the *in vivo* regulation can actually lead to small phenotypic changes, such as a slight increase weight and improved OGTTs during a 10 week HFD challenge upon comparing to the adiponectin KO littermate controls. No difference was observed in ITT and triglyceride clearance test (**Suppl. Fig. 2**).

We have previously reported that in the absence of insulin, adiponectin is critical for lipid clearance (15). We therefore treated the TRE-apn/KO animals with streptozotocin (STZ) and confirmed the presence of the induced adiponectin functionally with a triglyceride clearance test. Upon removal of insulin, the full potential of adiponectin is unleashed, and even the low level of adiponectin in TRE-apn/KO in the absence of insulin is sufficient to protect the animals from enhanced wasting and to promote lipid uptake when compared to adiponectin KO littermate controls under the same conditions (**Suppl. Fig. 3**).

Streptozotocin treatment and denervation can enhance adiponectin expression

Upon STZ treatment, not only can we observe an improvement in lipid uptake during a triglyceride clearance test, we also observe an increase in adiponectin mRNA expression due to increased transgene levels as judged by ELISA from TRE-apn/KO animals (**Suppl. Fig. 3a, Figs. 4a-b**). This observation was further validated by western blotting following immunoprecipitation of 100µl plasma samples collected from TRE-apn/KO and adiponectin KO with or without (control) STZ treatment (**Fig. 4c**).

We wondered what other effects could explain the discrepancy between the *in vitro* and the *in vivo* results. One of the most discerning differences in the *in vitro* settings is the lack of innervation. We first transplanted sWAT isolated either from an adiponectin KO or a TRE-apn/KO into an adiponectin KO recipient animal. Within 48 hours, we were able to observe increased transgene induction accompanied by increased adiponectin mRNA in the transplanted sWAT from TRE-apn/KO (**Fig. 4d**). Similarly, local denervation of fat pads *in situ* with 6-hydroxydopamine can increase adiponectin expression in the sWAT of TRE-apn/KO (**Fig. 4e**). However, none of the procedures was able to restore adiponectin expression to the full extent comparable to levels seen in WT.

Discussion

Prior publications have reported on overexpressing adiponectin globally, constitutively, or through adenoviral mediated overexpression (16-18). Attempts to overexpress adiponectin from adipocytes necessitated the introduction of a deletion in the collagenous stalk of adiponectin in order to enhance the release of adiponectin from adipocytes, leading to a robust 2-4 fold increase of adiponectin in circulation (18). Other attempts to overexpress adiponectin from an adipocyte-specific promoter also lead to a net decrease in adiponectin production (19,20).

To study this phenomenon more closely and to avoid any developmental issues, we generated a mouse model allowing for inducible, cell-specific overexpression. Our system is able to deliver up to 600-fold increase in plasma adiponectin when overexpressed from hepatocytes, hence demonstrating the effectiveness of this construct. This liver-derived adiponectin is at least partially functional. However, liver derived adiponectin does display differential modifications, apparent by a slight shift in molecular weight in western blots, and a very different half-life that leads to massive accumulation of the protein in serum. Our target cell-specific adiponectin overexpression model allows us to deliver adiponectin locally and at any desired time point during the pathogenic response to a challenge. For instance, we previously reported on adiponectin's anti-fibrotic effect using a global, constitutively expressed truncated adiponectin model in combination with lentivirus encoding adiponectin to dissect out its regulatory mechanism in hepatic stellate cells (18,21). With our current system, we are able to compare the cell autonomous and non-autonomous effects of adiponectin in a number of different cell types, including hepatic stellate cells and in the renal tubular epithelium.

On the other hand, we observed a number of intriguing phenomena when we induced the same transgene in adipocytes. In the presence of endogenous adiponectin, the transgene induction resulted in a reduction of plasma adiponectin levels despite a 3-fold increase in the total mRNA levels in response to the transgene. A previous study using ap2 promoter driven adiponectin overexpression already reported a self-regulatory feedback loop at both the transcription and the translation levels. In their follow up study, they overcame the feedback loop by selecting a line carrying 100 copies of the transgene to achieve a 2.5 fold increase in

mRNA level resulting in a 2-fold increase at the protein level (19,20). Our adipocyte-specific overexpression model is in a more physiological range. We were able to achieve a 3-fold overexpression at the transcriptional level, yet even at this lower level of overexpression, we still observe the tight regulation of adiponectin production. We took an additional approach to examine the regulation of this feedback loop. We first tested whether the endogenous adiponectin production limited the capacity of the adipocyte to overexpress adiponectin. We bred the TRE-apn/WT first into adiponectin heterozygous background then interbred into adiponectin KO background (TRE-apn/KO). Under both conditions, the animals failed to produce ectopic adiponectin at a significant level despite a clear transgene induction at the mRNA level. This indicates that the adipocyte-specific regulation is independent of the level of endogenous adiponectin. Nonetheless, the small fraction of adiponectin that escaped from the regulatory machinery is sufficient to exert beneficial effects when challenged with a HFD. Upon STZ-treatment, the TRE-apn/KO animals were further confirmed to produce functional adiponectin, though only at low levels.

Further insights into this regulatory system came from the *in vitro* studies using differentiated adipocytes isolated from the SVF of TRE-apn/KO mice. First, the fact that we were able to observe normal adiponectin expression at the protein level both in the secreted and intracellular forms suggests that the regulation is not cell-autonomous and only observed in the *in vivo* setting. Secondly, despite the 3-fold increase in adiponectin mRNA levels compared to wildtype *in vitro*, this overexpression can only achieve comparable but not higher adiponectin production when compared to the differentiated adipocytes cultured from WT animals under the same conditions. This suggests that the absolute amount of adiponectin mRNA *per se* is not rate limiting for adiponectin production. This is not surprising, since the mRNA for adiponectin is already one of the most abundant mRNAs in the adipocyte, and numerous instances have shown that an increase in circulating adiponectin does not necessitate an increase in its mRNA (22,23)

We examined the main differences between the *in vivo* setting and the *in vitro* situation. Adipose tissue innervation affects adipocyte function (24). Adipocyte function may be centrally regulated through neuronal mechanisms. While many adipocyte-derived factors are

released by “mass action”, i.e. the more adipose tissue there is, the more of these factors are released by an individual fat pad, the situation for adiponectin is quite different. Adiponectin levels are extremely stable over the short term, and reduced fat mass tends to trigger increased adiponectin in circulation. This necessitates coordination between the fat pads, and hence a neuronally-mediated mechanism may be necessary for its regulation. As a result, denervating the adipose tissues allowed us to test whether this has an impact. We denervated the sWAT by grafting it into an adiponectin KO recipient. In addition, we chemically denervated the sWAT while leaving the vasculature intact. Under both conditions, we can observe a significant enhancement in terms of mRNA expression, but still fail to normalize adiponectin levels. Similarly, STZ treatment only marginally increases adiponectin protein expression. Therefore, insulin signaling and adipose tissue innervation contribute in part to this regulatory machinery, but cannot explain the full nature of repressive mechanism, suggesting that these two pathways may be secondary to a broader control.

We believe at this point that the regulation is most likely post-transcriptional in its nature. Our work has taken a major step forward in dissecting the regulatory mechanism on adiponectin secretion and laid down the foundation for the future to study the key mechanisms responsible for this unique regulatory event for adiponectin in the adipocyte.

Author Contributions

C.T. and P.E.S. designed the experiments and wrote the manuscript. K.S. generated the TRE-apn animal, C.T., Q.A.W., R.Y., X.L., S.H., X.Y. performed animal experiments and analyzed data. Q.A.W. and R.K.G. performed the in vitro studies.

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Figures

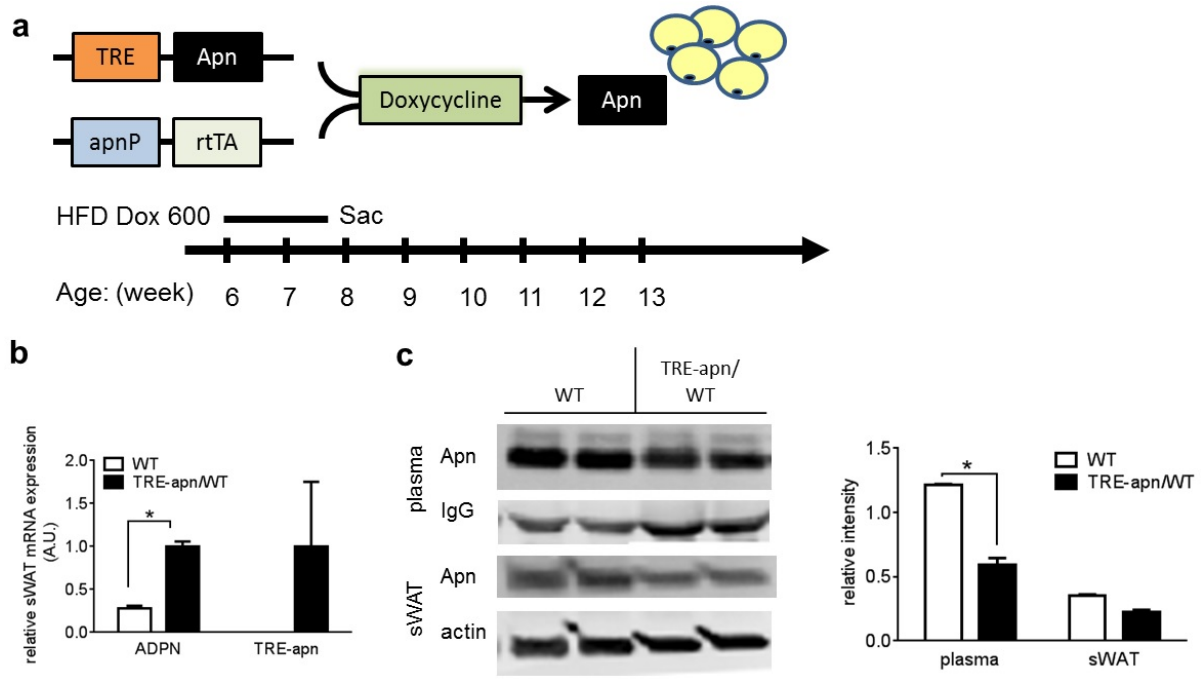


Figure 1: Ectopic overexpression of adiponectin from adipocytes results in reduced plasma adiponectin levels

a) schematic model of doxycycline inducible adipose tissue specific overexpression timeline Dox treatment for TRE-apn/WT animals.

b) qPCR analysis of endogenous and exogenous adiponectin mRNA expression in the subcutaneous white adipose tissue (sWAT) normalized to TRE-apn/WT after 10 days Dox induction.

c) Western blot of adiponectin in plasma and the sWAT isolated from 8 week old WT and TRE-apn/WT mice, on 10 days HFD Dox 600. Relative intensity is normalized to either IgG or actin signal.

*p<0.0 5 compared to WT. Data are presented as means ± SEM. n=3

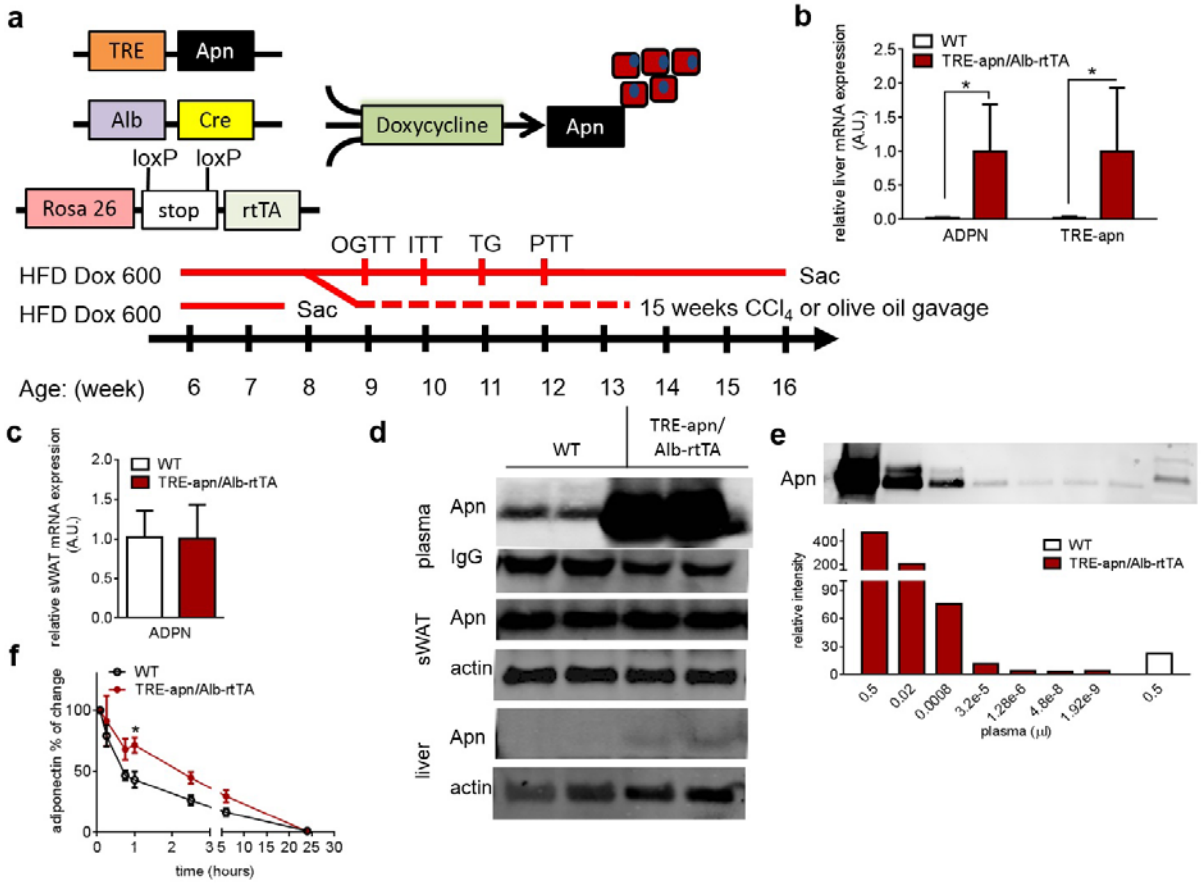


Figure 2: Adiponectin overexpression from hepatocytes, in contrast to adipocytes, gives rise to high circulating adiponectin levels

a) schematic model of doxycycline inducible hepatocyte specific overexpression timeline of Dox treatment and metablic tests for TRE-apn/Alb-rtTA animals.

b-c) qPCR analysis of endogenous and exogenous adiponectin mRNA expression in the liver (b) and sWAT (c) normalized to TRE-apn/Alb-rtTA after 10 days Dox induction.

d) Western blot of adiponectin in plasma, perfused sWAT and liver isolated from 8 week old WT and TRE-apn/Alb-rtTA mice, on 10 days HFD Dox 600.

e) adiponectin western blot using serial diluted plasma samples from 10 days HFD Dox treated TRE-apn/Alb-rtTA mice .

f) decay curve of endogenous or liver-derived adiponectin by tail vein injection of 100ng adiponectin from WT or TRE-apn/Alb-rtTA adiponectin into adiponectin knockout mice. *p<0.0 5 compared to WT. Data are presented as means ± SEM n=3

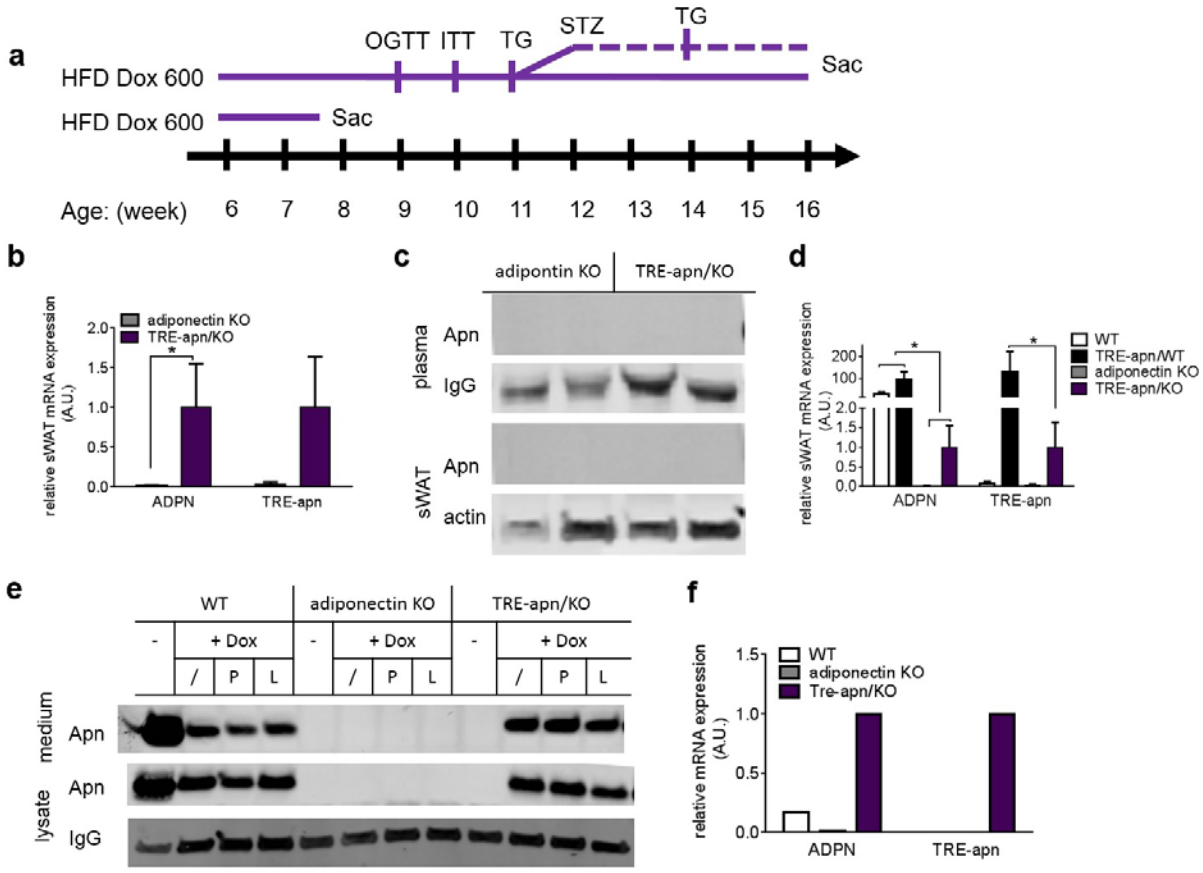


Figure 3: Adipocyte specific inhibition of ectopic adiponectin overexpression in vivo

a) timeline of Dox treatment and metablic tests for TRE-apn/KO animals .

b) qPCR analysis of endogenous and exogenous adiponectin mRNA expression in the sWAT normalized to TRE-apn/KO after 10 days Dox induction.

c) Western blot of adiponectin in plasma and sWAT isolated from 12 week old adiponectin KO mice and TRE-apn/KO mice on 10 weeks HFD Dox.

d) comparison of endogenous and exogenous adiponectin mRNA expression in the sWAT from WT, TRE-apn/WT, adiponectin KO, and TRE-apn/KO normalized to TRE-apn/KO after 10 weeks HFD Dox challenge.

e) adiponectin western bolt using medium and cell lysate collected from differentiated adipocytes cultured using s.v.f. of sWAT isolated from 6 week old WT, adiponectin KO, and TRE-apn/KO mice 24 hr after Dox(+) treatment (P=proteasome inhibitor MG132, L=lysosome inhibitor NH_4Cl).

f) qPCR analysis of endogenous and exogenous adiponectin mRNA expression from *in vitro* differentiated adipocytes normalized to TRE-apn/KO.

*p<0.05 compared to adiponectin KO and as indicated. Data are presented as means ± SEM

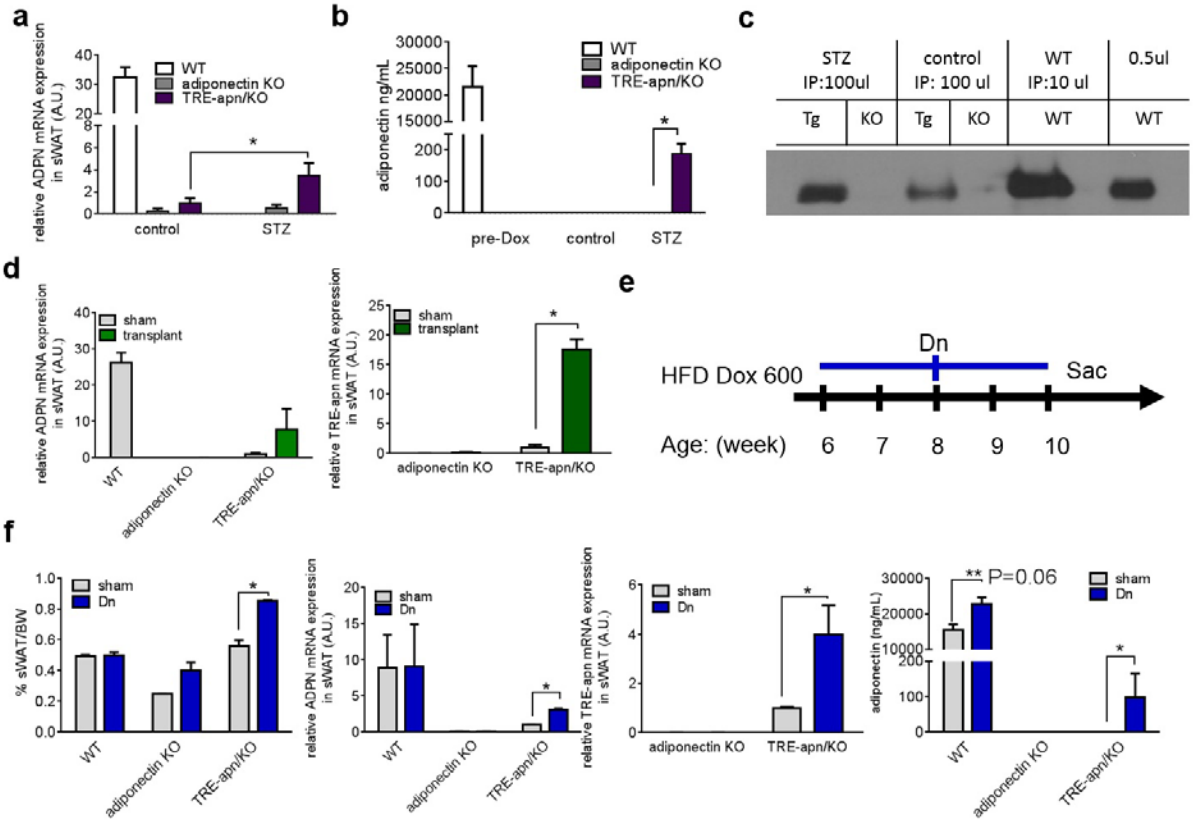


Figure 4: Streptozotocin treatment and denervation can enhance adiponectin expression

a) qPCR analysis of endogenous and exogenous adiponectin mRNA expression in the sWAT before and after STZ treatment normalized to TRE-apn/KO after 10 weeks HFD Dox challenge.

b) plasma adiponectin level before and after STZ treatment measured by adiponectin ELISA.

c) adiponectin western blot using 100 ul plasma from STZ treated and control TRE-apn/KO after immunoprecipitation with adiponectin antiserum.

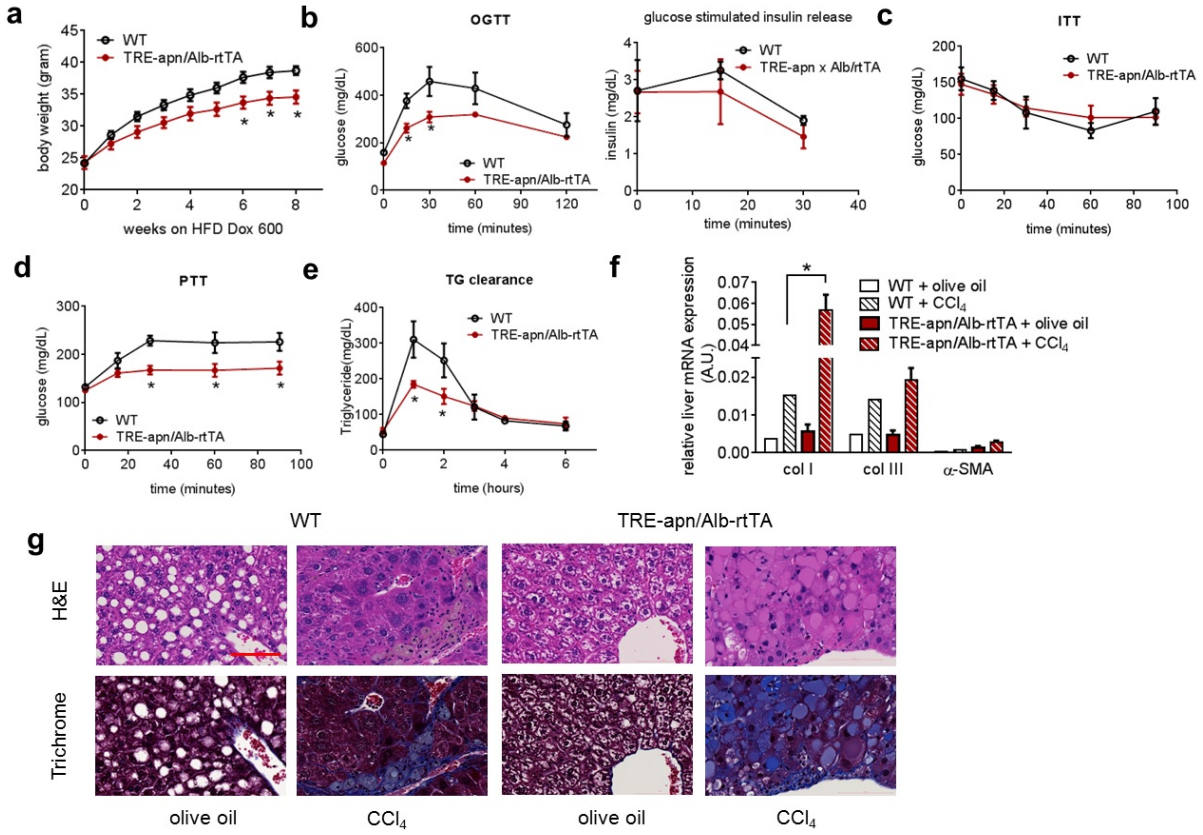
d) qPCR analysis of endogenous and exogenous adiponectin mRNA expression in the sWAT before and 2 days after transplantation, normalized to TRE-apn/KO.

e) timeline of Dox treatment for the adipose tissue denervation.

f) % sWAT to body weight, qRCR analysis of endogenous and exogenous adiponectin. mRNA expression normalized to TRE-apn/KO after 2 weeks of sWAT local denervation in WT, adiponectin KO and TRE-apn/KO.

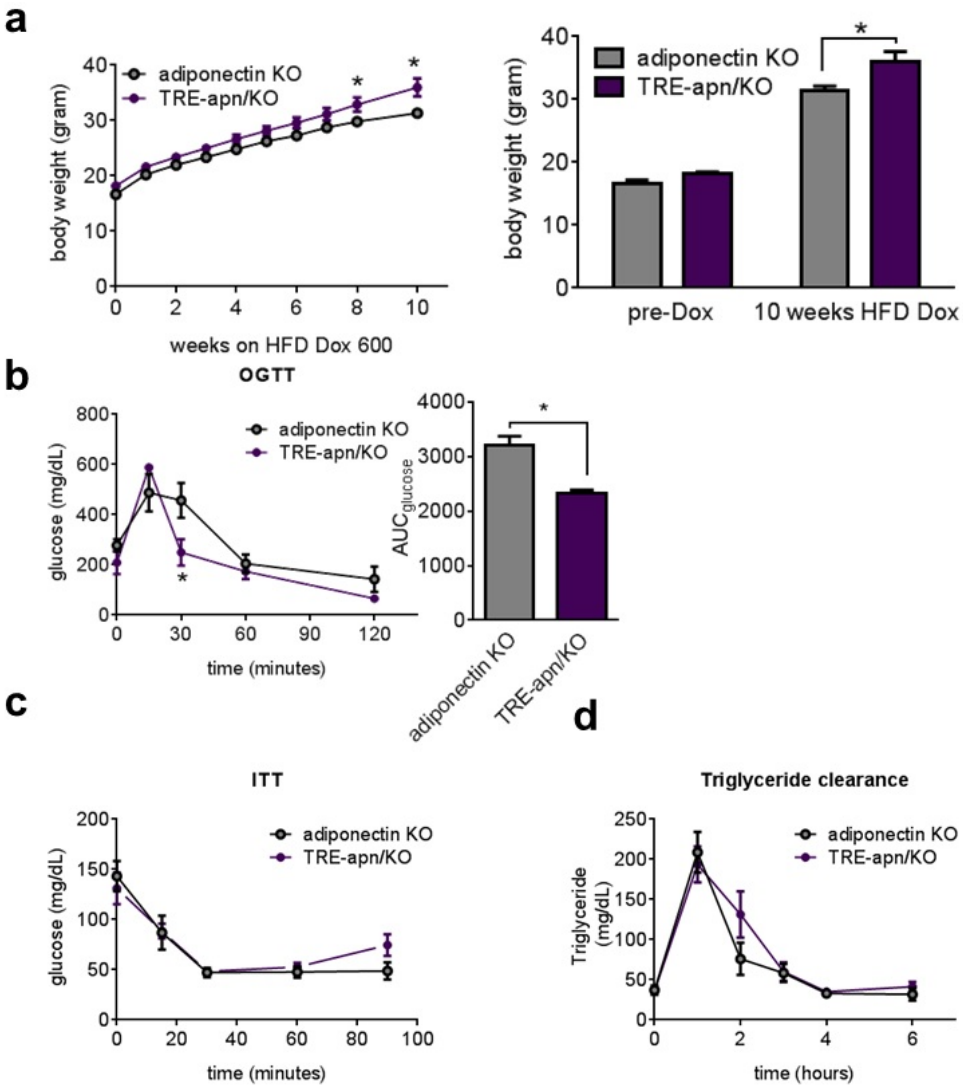
*$p<0.05$ as indicated. Data are presented as means ± SEM. n=3

Supplemental Figures



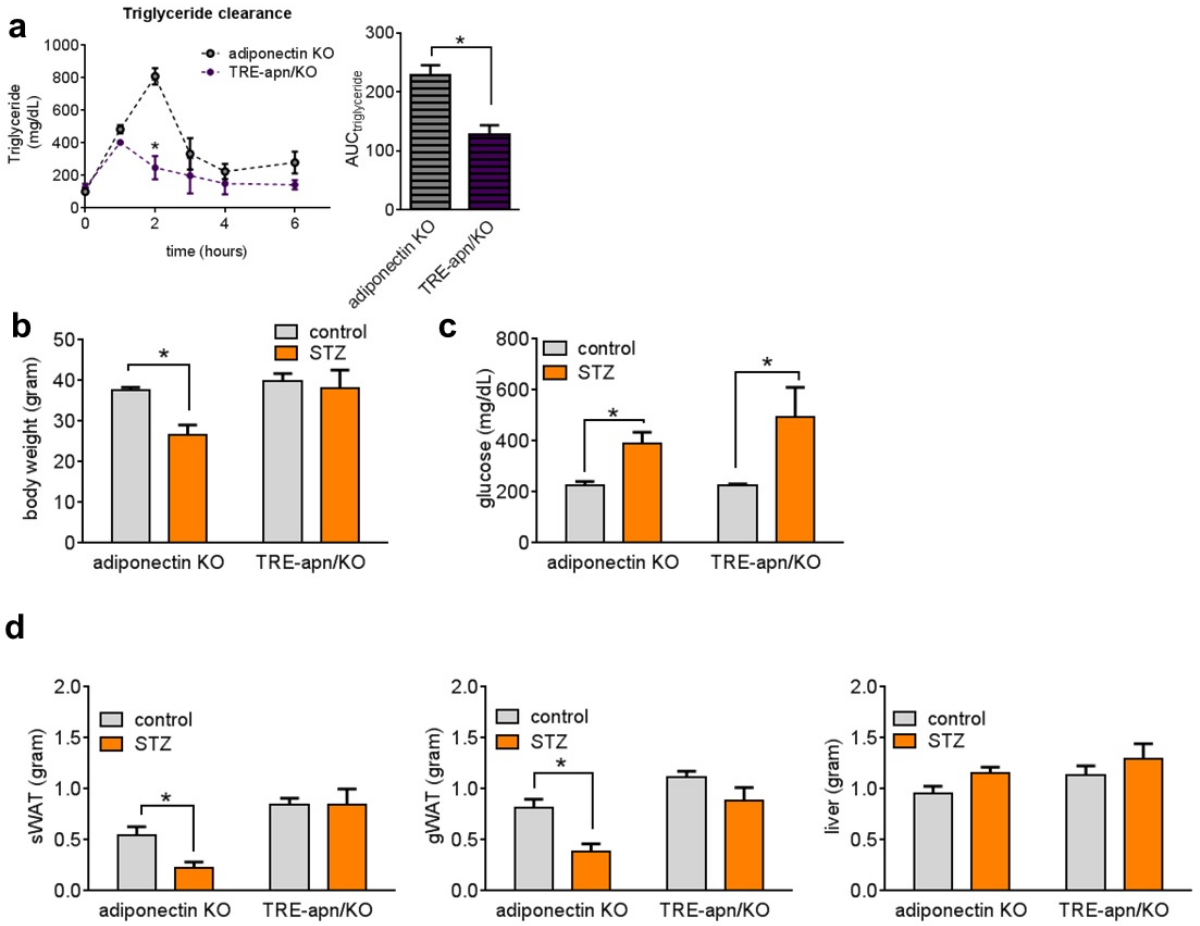
Supplement Figure 1: Hepatocytes overexpression improves glucose metabolism and is not protected from CCl_4 induced liver fibrosis

a) body weight curve in WT and TRE-apn/Alb-rtTA mice on HFD Dox 600 for 8 weeks (n=6-10).
b) oral glucose tolerance test and glucose stimulated insulin release during first 30 minutes (2.5 mg/g BW).
c) insulin tolerance test (0.75 mU/g BW).
d) pyruvate tolerance test (2 mg/kg BW).
e) triglyceride clearance test (15 ml/Kg BW of 20% intralipid) (n=4-8).
f) qPCR analysis of mRNA expression in the liver isolated from either olive oil or CCl_4. treated WT or TRE-apn/Alb-rtTA animals normalized to TRE-apn/Alb-rtTA treated with CCl_4 (n=3).
g) H&E (top) and trichrome stain (bottom) of WT and TRE-apn/Alb-rtTA liver. Animals received either 40% CCl_4 or olive oil (vehicle) through oral gavage twice a week for 15 weeks following two weeks of HFD Dox treatment. (magnification:10x scale bar 100um).
*p<0.05 compared to WT. Data are presented as means ± SEM



Supplement Figure 2: Adipocytes overexpression improves glucose sensitivity after HFD challenge

a) body weight and body weight gain in adiponectin KO and TRE-apn/KO mice during 10 weeks HFD Dox 600 challenge (n=15-16).
b) oral glucose tolerance test (2.5 mg/g BW).
c) insulin tolerance test (0.75 mU/g BW).
d) triglyceride clearance test (15ml/kg BW of 20% intralipid) (n=4).
*p<0.05 compared to adiponectin KO. Data are presented as means ± SEM



Supplement Figure 3: Adipocytes overexpression protect animals from loss in adipose mass and promote lipid uptake in the absence of insulin

a) triglyceride clearance test (15ml/kg BW) after STZ treatment (135ug/g BW) (n=3).
b-d) body weight (b), plasma glucose (c) and tissue weight (sWAT, gWAT and liver).
(d) from control and STZ treated TRE-apn/KO and adiponectin KO animals (n=4-5, 10 week post HFD Dox 600).
*$p<0.05$ as indicated. Data are presented as means ± SEM

Supplemental Table

Supplemental table 1: Primer sets for qPCR analysis

Gene	Forward primer	Reverse primer
α-SMA	ACTGGGACGACATGGAAAAG	CATCTCCAGAGTCCAGCACA
ADPN	GTTGCAAGCTCTCCTGTTCC	ATCCAACCTGCACAAGTTCC
β-actin	TACCACAGGCATTGTGATGG	TTTGATGTCACGCACGATTT
collagen I	GAGCGGAGAGTACTGGATCG	TACTCGAACGGGAATCCATC
collagen III	TGGTCCTCAGGGTGTAAAGG	GTCCAGCATCACCTTTTGGT
TRE-apn	GGGGACCACAATGGACTCTA	TTTGCCCCCTCCATATAACA

Methods

Mice

Mice were maintained on a 12 hour dark/light cycle and housed in groups of three to five with unlimited access to water, chow (No. 5058, Lab-Diet) or high fat (60% of calories from fat) doxycycline containing diet (600mg kg^{-1}) (HFD Dox)(S4107, Bio-Serv) as indicated for the individual experiments. The Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center, Dallas, has approved all animal experiments. TRE-Apn transgenic mice were generated on pure C57B6/J background by the UTSW Transgenic Core Facility. apnP-rtTA and Apn KO mouse was generated as previously described(23,24) Alb-Cre, GFAP-Cre, and Rosa26-loxP-STOP-rtTA lines were purchased from the Jackson Laboratories on FVB background. For liver overexpression, TRE-Apn were backcrossed a minimum of 10 times into the FVB background, then intercrossed with Alb-Cre or GFAP-Cre and Rosa26-loxP-STOP-rtTA.

Generation of transgenic mice

To generate a Dox-inducible adiponectin overexpression mouse model (TRE-Apn), the complementary DNA encoding the full-length murine adiponectin (amino acids 115–858, NM_009605.4) was subcloned into the pBluescriptTRE tight vector (Clontech). To better stabilize the transcript and enhance the translation, a Kozak sequence 5′-GCCGCCACC-3′ was inserted in front of the start codon and the rabbit β-globin 3′UTR was introduced into the 3′ end of the vector(25). After linearization, TRE-Apn DNA was injected to embryos of a C57BL/6 background by the Transgenic Core Facility at UT Southwestern Medical Center.

Transplantation of sWAT

Fat transplantation was performed as previously described (26). Donor and recipient mice were age- and sex-matched to minimize rejection. sWAT (500 mg) from the donor was inserted into the subcutaneous space on the back of the recipient. The amount of WAT transplanted represented about 30% of the total sWAT weight in chow-fed C57BL/6 mice. After 48 hours, the grafts were dissected out and evaluated via qPCR.

Local sympathetic nervous system denervation

Local sympathetic nervous system denervation was performed as previously described (27). The left sWAT of each animal received 12 small 2 µl injection of 6-hydroxydopamine (9mg/ml in saline containing 1% ascorbic acid). The right sWAT was injected with the vehicle under the same surgical condition. Body weights of the mice were recorded everyday day for two weeks. At the end of the time course, all WAT were rapidly dissected, weighed and snap frozen in liquid nitrogen.

Western blot analysis

Adipose tissue samples were homogenized on ice in T-PER (Thermo Scientific, USA) supplemented with protease inhibitor cocktail (cOmplete mini, EDTA-free, Roche). This was followed by low speed centrifugation (3,000xg at 4°C), in order to remove the fat cake from the top. The tissue extracts were cleared at 15,000 x g for 20 minutes at 4°C and total protein concentration measured with BCA Protein Assay Kit (Pierce Protein Research Products, Thermo Scientific, USA). Equal amounts of protein from adipose tissue were mixed with 5x Laemmli sample buffer with DTT and boil at 95°C for 5 minutes. The samples were resolved on 4-15% TGX (Bio-Rad, USA), followed by semi-dry transfer to PVDF membrane (Bio-Rad, USA). Blots were probed with rabbit polyclonal anti mouse adiponectin antibody(4). Bound antibodies were detected with IRDye800-conjugated anti-rabbit secondary antibodies (Rockland). Membranes were scanned with the LI-COR Odyssey Infrared Imaging System. Actin controls were visualized with an anti-mouse actin monoclonal antibody from Cell Signaling with IRDye680-conjugated anti-mouse secondary antibodies (Rockland).

Blood biochemistry

Insulin and adiponectin levels were measured by commercial ELISA kits from Millipore (Billerica, MA, USA). Glucose levels were determined with Sigma Diagnostics Glucose Reagents (Sigma, Sigma Aldrich, USA), Triglycerides were measured using Infinity Triglycerides Reagent (Thermo Scientific, USA) and free fatty acids levels were measured with NEFA-HR(2) (Wako Pure Chemical Industries, Japan).

Isolation and differentiation of adipose stromal vascular cultures

Dissected fat tissue from two mice (4 fat pads total) were washed, minced, and then digested for two hours at 37ºC in buffer containing 100mM HEPES pH 7.4, 120mM NaCl, 50mM KCl, 5mM glucose, 1 mM $CaCl_2$, 1.5% BSA, and 1mg/mL collagenase D (Roche). Digested tissue was then filtered through a 100 μm cell strainer to remove undigested fragments. The flow-through was then centrifuged for 5 minutes at 600 x g to pellet the SV cells. The SV cells, resuspended in complete SV culture medium (DMEM/F12) (Invitrogen) plus Glutamax, Pen/Strep, and 10% FBS), were then filtered through a 40μm cell strainer to remove clumps and large adipocytes. Following centrifugation as above, SV cells were then resuspended in SV culture medium and plated onto a 6-cm tissue culture dish. For adipocyte differentiation assays, SV cells were plated onto collagen-coated dishes and grown to confluence in SV culture medium. At confluence, cells were exposed to the adipogenic cocktail containing dexamethasone (1μM), insulin (5μg/ml), isobutylmethylxanthine (0.5mM) (DMI) and rosiglitazone (1μM) in SV culture medium. Forty-eight hours after induction, cells were maintained in SV culture medium containing insulin (5μg/ml) and rosiglitazone (1μM) until harvest.

Oral glucose and insulin tolerance tests

Oral glucose tolerance test (OGTT) was performed in mice on HFD Dox for 3 weeks starting at 6 weeks of age. One week later, the same mice were used for insulin tolerance test (ITT). For OGTT, mice were fasted for 4 hour during light phase and blood samples were drawn from tail vein before and 15, 30, 60, and 120 minutes after oral gavage with 2.5 mg/g BW of glucose in PBS. For ITT, 4-h fasted mice were injected intraperitoneally with recombinant human insulin

(Novo Nordisk, Inc.) at 0.75 mU/g BW and blood samples were collected before and 15, 30, 60, and 90 minutes after.

Triglycerides clearance

Triglycerides clearance (TG clearance) was performed in mice on HFD Dox for 5 week starting at 6 weeks of age. The mice were fasted for 6 hour during light phase and blood samples were drawn from tail vein before and 1, 2,3,4,6 hours after oral gavage with 15ml/kg BW of 20% intralipid (Fresenius Kabi Clyton, L.P.).

Quantitative real-time RT-PCR

Tissues were snap freeze in liquid nitrogen and stored at -80°C. Trizol Reagent (Invitrogen, USA) extraction followed by RNA purification using RNeasy Mini Kit and RNase-Free DNase (Qiagen, USA). RNA was reverse transcribed to cDNA by iScript cDNA synthesis kit (Bio-Rad, USA) and SYBR Green (Applied Biosystems, life technologies, USA) was used for the quantitative PCR reactions. The relative expression level was calculated by the comparative Ct method using β-actin as endogenous control. Primer sequences can be found in **Supplemental Table 1**.

Statistical Analysis

All the results are presented as means ± Standard Error of the Mean (SEM). Differences between two groups were determined for statistical significance by a standard two-tailed Student t test. Significance was accepted at a value of $p < 0.05$.

(The following section is manuscript prepared for submission as an original basic research article)

2:2 ADIPONECTIN PREVENTS THE DEVELOPMENT OF CARBON TETRACHLORIDE INDUCED-LIVER FIBROSIS THROUGH LOCAL HEPATIC STELLATE CELLS OVEREXPRESSION USING THE GFAP PROMOTER

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Author contributions

C.T. and P.E.S. designed the experiments and wrote the manuscript. K.S. generated the TRE-apn animal, C.T., Q.A.W., Y.D, C.Y. performed animal experiments and analyzed data. K.LP. assist with the imaging analysis

Acknowledgements

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Ethics approval by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center, Dallas

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Abstract

Liver is a vital organ due to its role in carbohydrate, lipid and amino acid metabolism and the synthesis of essential proteins. Dysfunction or injury to the liver affects mortality and morbidity. Finding preventive and diagnostic measures for liver disease is an ongoing effort and clinically an area of high need. Adiponectin has potent insulin sensitizing effects and decreases hepatic glucose production and decreases vascular inflammation. Here, we summarize our current findings on local adiponectin overexpression in response to carbon tetrachloride-induced liver fibrosis with the hope to use it as a preventive measure in the future.

AIM: To determine the effect of local hepatic stellate cells adiponectin in the development of steatohepatitis.

METHOD: Target-cell specific adiponectin overexpression was induced in hepatic stellate cells (HSCs) under the control of the glial fibrillary acidic protein (GFAP) promoter. Liver sections from animals that received either 40% CCl_4 or olive oil (vehicle) through oral gavage twice a week for 15 weeks following two weeks of HFD Dox treatment were examined for fibrosis markers and collagen deposits.

RESULTS: Mice overexpressing adiponectin in HSCs can alter glucose and lipid metabolism during a HFD challenge. In addition, adiponectin overexpression in HSCs can protect the animals from CCl_4 induced liver fibrosis.

CONCLUSION: GFAP promoter-driven adiponectin overexpression can exert potent protective effects against CCl_4-induced liver fibrosis. By comparing the results with our previous hepatocyte overexpression models, we hypothesized that local adiponectin treatment in HSCs can stabilize HSCs in the quiescent state and prevent the initiation of fibrogenesis and the subsequent development of steatohepatitis.

Key words: adiponectin, insulin resistance, obesity, liver fibrosis

Core tip: Previously, adiponectin have been demonstrated to exert anti-fibrotic effect in other health condition. *In vitro* experiment using primary hepatocytes isolated from adiponectin knockout and adiponectin overexpression mice suggested potential role of adiponectin in the activation of HSCs. In this report, we examined the effect of adiponectin local overexpression in the development of liver fibrosis using a target-cell specific inducible overexpression model. Specifically, we compared the effects of hepatocyte-specific overexpression with overexpression in HSCs and found that only HSCs-specific overexpression of adiponectin can prevent the development of liver fibrosis.

Introduction

Since the initial discovery in 1995, research on adiponectin has greatly shaped how we view adipose tissue and highlight the importance of adipose tissue as an endocrine organ (1,2). Adiponectin, an adipocyte-specific secretory protein, has potent insulin sensitizing effects and affects glucose and fatty acid metabolism on peripheral tissues. Two receptors have been cloned from skeletal muscle (AdipoR1) and the liver (AdipoR2) (3). Numerous studies have demonstrated adiponectin's insulin sensitizing properties through receptor-mediated activation of the AMPK pathway (4,5). Consistent with the observed decreasing circulating adiponectin levels in obese patient cohorts, adiponectin is crucial for central and peripheral metabolic homeostasis (6-9). Recent publications have suggested additional evidence for the systemically beneficial effects of adiponectin (10,11). Here, we focus specifically on the pleiotropic actions of adiponectin in the liver.

Liver disease can be caused by a spectrum of factors, including genetic mutations, ectopic lipid storage, and viral infection (12). One in ten Americans are affected by liver disease (13). Low plasma adiponectin has been found to associate with numbers of liver disease states. Animal studies using recombinant adiponectin have demonstrated its potential to improve the condition of non-alcoholic fatty liver disease and alcoholic liver disease (14,15). Hepatic steatosis is considered a risk factor for advanced liver disease; however, only a fraction of the affected individuals continue to develop steatohepatitis. In rodent models, it is very challenging to induce a fibrotic phenotype in the liver unless the mice are exposed to extreme challenges (16,17). Steatohepatitis is characterized by immune infiltration and hepatocyte apoptosis. Depending on the degree of injury, fibrogenesis may be partially initiated.

Fibrogenesis is a complex wound-healing response involving multiple aspects, such as nuclear receptors, inflammatory cytokines, and growth factors. Prolonged fibrogenesis can ultimately lead to fibrosis and cirrhosis. Fibrosis, or scarring tissue, is characterized by an accumulation of fibrillar collagen (collagens I and III) in the extracellular matrix (ECM). It has been shown that activation of hepatic stellate cells (HSCs) and Kupffer cells (KCs) can modulate hepatic fibrogenesis in injured liver tissue (18). Not all, but most of the fibrotic cells are derived from activated HSCs (17,19). Upon liver injury, quiescence HSCs lose partial

structural integrity and their lipid droplet function (where they store retinol) to switch on collagen production and become more proliferative. Activated HSCs are fibrogenic and feedback on other HSCs that they prompt to activate via amplifying the inflammatory response (16,20,21).

Thus far, numerous groups have focused their attention on reversing the fibrosis condition. Here, we offer the potential of using adiponectin in local HSCs as a preventive measure for the progression of fibrosis from simple steatosis.

Materials and Methods

Mice

Mice were maintained on a 12 hour dark/light cycle and housed in groups of three to five with unlimited access to water, chow (No. 5058, Lab-Diet) or high fat (60% calories from fat) doxycycline containing diet (600mg kg^{-1}) (HFD Dox)(S4107, Bio-Serv) as indicated for the individual experiments. The Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center, Dallas, has approved all animal experiments. TRE-Apn transgenic mice were generated on pure C57B6/J background by the UTSW Transgenic Core Facility, GFAP-Cre, and Rosa26-loxP-STOP-rtTA lines were purchased from the Jackson Laboratories on a FVB background. TRE-Apn were backcrossed a minimum of 10 times into the FVB background, then intercrossed with GFAP-Cre and Rosa26-loxP-STOP-rtTA.

Carbon tetrachloride induced fibrosis

Animals received either 40% CCl_4 or olive oil (vehicle) through oral gavage twice a week for 15 weeks following two weeks of HFD Dox treatment.

Western blot analysis

Adipose tissue samples were homogenized on ice in T-PER (Thermo Scientific, USA) supplemented with protease inhibitor cocktail (*cOmplete mini*, EDTA-free, Roche). This was followed by low speed centrifugation (3,000xg at 4°C), in order to remove the fat cake from the top. The tissue extracts were cleared at 15,000 x g for 20 minutes at 4°C and total protein concentration measured with a BCA Protein Assay Kit (Pierce Protein Research Products, Thermo Scientific, USA). Equal amounts of protein from adipose tissues were mixed with 5x Laemmli sample buffer with DTT and boiled at 95°C for 5 minutes. The samples were resolved on 4-15% TGX (Bio-Rad, USA), followed by semi-dry transfer to a PVDF membrane (Bio-Rad, USA). Blots were probed with a rabbit polyclonal anti-mouse adiponectin antibody (2). Bound antibodies were detected with IRDye800-conjugated anti-rabbit secondary antibodies (Rockland). Membranes were scanned with the LI-COR Odyssey Infrared Imaging System. Actin controls were visualized with an anti-mouse actin monoclonal antibody from Cell Signaling with IRDye680-conjugated anti-mouse secondary antibodies (Rockland).

Blood biochemistry

Insulin was measured with a commercial ELISA kit from Millipore (Billerica, MA, USA). Glucose levels were determined with Sigma Diagnostics Glucose Reagents (Sigma, Sigma Aldrich, USA), Triglycerides were measured using Infinity Triglycerides Reagent (Thermo Scientific, USA) and free fatty acids levels were measured by NEFA-HR (2) (Wako Pure Chemical Industries, Japan).

Oral glucose and insulin tolerance tests

Oral glucose tolerance tests (OGTTs) were performed in mice after HFD Dox for 3 weeks starting at 6 weeks of age. One week later, the same mice were used for insulin tolerance tests (ITT). For OGTTs, mice were fasted for 4 hours during the light phase and blood samples were drawn from the tail vein before and 15, 30, 60, and 120 minutes after oral gavage with 2.5 mg/g BW of glucose in PBS. For ITTs, 4-h fasted mice were injected intraperitoneally with recombinant human insulin (Novo Nordisk, Inc.) at 0.75 mU/g BW and blood samples were collected before and 15, 30, 60, and 90 minutes after injection.

Triglyceride clearance

Triglycerides clearance (TG clearance) was performed in mice on HFD Dox for 5 week starting at 6 weeks of age. The mice were fasted for 6 hours during light phase and blood samples were drawn from tail vein before and 1, 2, 3, 4, 6 hours after oral gavage with 15 ml/kg BW of 20% intralipid (Fresenius Kabi Clyton, L.P.).

Quantitative real-time RT-PCR

Tissues were snap freeze in liquid nitrogen and stored at -80°C. Trizol Reagent (Invitrogen, USA) extraction followed by RNA purification using RNeasy Mini Kit and RNase-Free DNase (Qiagen, USA). RNA was reverse transcribed to cDNA by iScript cDNA synthesis kit (Bio-Rad, USA) and SYBR Green (Applied Biosystems, life technologies, USA) was used for the quantitative PCR reactions. The relative expression level was calculated by the comparative Ct method using β-actin as endogenous control. Primer sequences can be found in **Supplemental Table 1**.

Statistical Analysis

All the results are presented as means ± Standard Error of the Mean (SEM). Differences between two groups were determined for statistical significance by a standard two-tailed Student t test. Significance was accepted at a value of $p < 0.05$

Results

Overexpression of adiponectin from HSCs

Previously, we reported our studies on adiponectin local overexpression using target cell specific and doxycycline (Dox)-inducible adiponectin overexpressing transgenic animals. Briefly, we can achieve 600-fold higher circulating adiponectin compared to wildtype (WT) animals when we overexpress adiponectin in hepatocytes. The liver-derived adiponectin displays effects comparable to endogenous adiponectin during various metabolic characterizations. However, upon challenging the mice with carbon tetrachloride (CCl_4), the hepatocyte-overexpression animals develop more severe liver fibrosis compared to littermate controls receiving the same treatment. Here, we took the same TRE-apn transgene and overexpressed adiponectin under the control of the GFAP (Glial fibrillary acidic protein) promoter (**Fig. 1a**). The GFAP promoter is most widely used in studies in the central nervous system. We are currently in the process of making a better inducible HSCs specific model using the lecithin:retinol acyltransferase (LRAT) promoter, based on a study published by Mederacke et al. (22). However, the GFAP promoter also potently drives transcription in HSCs. In our current studies where we focus on the local effects of adiponectin only, we are not overly concerned about the fact that adiponectin is also expressed from the central nervous system. Our previous hepatocyte-overexpression model also serves as a control for the systemic effects of adiponectin overexpression. Furthermore, previous studies of adiponectin action in the brain already established the phenotype associated with adiponectin effects in the central nervous system (23). Therefore, we can reasonably well differentiate the local effects of adiponectin from the systemic effects.

After Dox induction, we observed significant adiponectin mRNA expression in the liver (**Fig. 1b**). This overexpression did not change the endogenous adiponectin production in the subcutaneous white adipose tissue (sWAT) at the transcriptional level. (**Fig 1c**). Similar to the hepatocyte-specific overexpression model, we detected high levels of circulating adiponectin from the HSC-specific overexpression model (**Fig. 1d**). The increase in adiponectin protein in the sWAT is primary due to the HSC-derived protein, not from increasing the endogenous adiponectin production, since adiponectin detected in serum has a

shift in molecular weight. Unlike the hepatocyte-derived model, there is a higher degree of adiponectin accumulation within the liver, which may hint at an increased intracellular accumulation of adiponectin with HSCs (**Fig. 1e**).

HSCs overexpression alters glucose and lipid metabolism

Under the control of the GFAP promoter, the excess adiponectin found in circulation potently protect against high fat diet (HFD) induced obesity (**Fig. 2a**) During an oral glucose tolerance test (OGTT), the overexpression animals had significantly reduced glucose-stimulated insulin release, while maintaining the same blood glucose level (**Fig. 2b**). They also displayed significant improvement during an insulin tolerance test (ITT) (**Fig. 2c**). One of the possible mechanisms accounting for the observed improvement in glucose homeostasis is that the excess adiponectin is an insulin sensitizer. The mice become more insulin responsive with the same amount of insulin, and a more robust response is initiated when compared to littermate controls.

The resistance to HFD-induced obesity may be mediated through decreased lipid uptake and increased fatty acid oxidation in the liver. After an oral intralipid gavage, the HSC adiponectin overexpressing animals display severely impaired lipid excursion (**Fig. 2d**). After six weeks of HFD, the HSCs overexpression mice began to display a weight loss phenotype. Six more weeks after the weight reduction, the animals lost almost all body fat. This weight loss effect was not observed previously in the hepatocyte-specific overexpressing model; this suggests that it is unlikely to be caused by the elevation of circulating adiponectin. It is more likely due to the local accumulation of the protein inside the liver. This weight loss phenotype may also partially be attributed by the effects of adiponectin expression in brain as observed in a previous study (23). However, we can rescue the body weight phenotype by supplying extra lipid through olive oil gave, every other day, in addition to HFD (**Fig. 2e**). This suggests that prolonged adiponectin accumulation in the liver can disturb lipid homeostasis, leading to lipodystrophy.

Adiponectin HSCs overexpression protects against CCl_4 induced liver fibrosis

It is fortunate that the lipodystrophy phenotype can be prevented through extra olive oil gavages. With this limitation in mind, we focused our attention on the local effects of

adiponectin in terms of liver fibrosis. One of the standard methods to induce liver fibrosis in rodent models is through oral administration of the hepatotoxin carbon tetrachloride (CCl_4). We mixed CCl_4 with olive oil and compared to littermates receiving olive oil gavage only. After 15 weeks, WT animals receiving olive oil only had increased steatosis as evidenced by the accumulation of larger lipid droplets in the liver. WT animals receiving CCl_4 showed collagen deposition after trichrome staining, suggesting the development of liver fibrosis. On the contrary, HSCs overexpression animals receiving CCl_4 did not have any positive collagen staining (**Fig. 3a**). qPCR analysis of fibrosis markers also showed reduced expression level in the HSCs overexpression animals after receiving CCl_4 compared to WT animals treated in the same condition (**Fig. 3b**). Previously, we reported that the hepatocyte-specific overexpressing animals displayed a deteriorated liver phenotype after CCl_4-induced liver damage. By comparing the two models side by side, we concluded that it is the HSCs local adiponectin overexpression, not the global adiponectin levels, that are critical in preventing fibrotic conditions.

Discussion

Activated HSCs are functionally and phenotypically distinct from quiescent HSCs. The activated HSCs are proliferative and are able to release paracrine factors and survival signals to promote expansion of progenitor cells, scarring and hepatic regeneration(19,21). HSC activation can be broken down into two phases: the initiation and the perpetuation phase (19). The initiation phase refers to the early injury recognition event, such as LPS/TLR 4 signaling activation, sensory responses to oxidative stress and apoptotic hepatocytes, and paracrine stimuli from KCs. These early events are targeted towards the quiescent HSCs to induce collagen production and lead to progressive changes of the ECM(19,24). The perpetuation phase is characterized by a functional change following the phenotypic change of HSCs. Depending on the extrinsic stimuli; HSCs can be prompted to 1. proliferate to recover the loss of cell body due to apoptotic hepatocytes; 2. contract to increase portal resistance; 3. migrate to sites of injury; 4. work in synergy with TFG-β and other pro-fibrogenic signals to promote fibrogenesis; 5.enhance inflammatory signals(19-21,25,26). It is these differential functions of HSCs that give them the ability to contribute to fibrogenesis, and they open the potential for these cells to serve as therapeutic targets (19,24).

Adiponectin can also suppress the expression of fibrogenic genes and mitogens (27). Previously, adiponectin has been reported to exhibit anti-inflammatory and anti-fibrogenic effects in the context of cardiovascular and gastrointestinal diseases (28,29). An additional observation in favor of adiponectin and its anti-fibrotic potential is that quiescent HSCs resemble adipocytes in many ways, including the ability to secrete adiponectin. However, upon activation, instead of adiponectin, HSCs show enhanced leptin production. Based on this observation, previous studies have demonstrated the potential of using adiponectin for regression of fibrogenesis (19,30,31). First and foremost, Shafiei et al. reported that adiponectin overexpressing transgenic mice receiving thioacetamide were resistant to fibrosis, compared to controls. In contrast, adiponectin knockout animals developed severe fibrosis (30). Summarizing these observations and adiponectin's anti-apoptotic function, we hypothesized that adiponectin is anti-fibrotic by "clamping" HSCs into the quiescent state to prevent activation and the subsequent release of pro-fibrogenic and pro-inflammatory factors.

In this report, the systematic phenotype following GFAP promoter-driven overexpression may be due to the effects of adiponectin in the central nervous system, and we have to bear that in mind when we interpret the systemic metabolic changes. However, HSC phenotype is more likely due to the induction of adiponectin in the HSCs. It is unclear how high the CNS-derived production of adiponectin is, and whether this material contributes at all to the circulating levels. Similar to our previous hepatocyte-overexpression model, the HSCs-derived adiponectin also shows differential post-translational modifications. The prolonged accumulation of adiponectin in the liver (which we clearly did not observed in the hepatocyte overexpression system) exerts detrimental effects on lipid homeostasis. These metabolically unfavorable changes can be rescued by supplying extra olive oil through an oral gavage, suggesting that the excessive local adiponectin may have depleted the intracellular triglyceride pool by restricting the uptake and potentially activating free fatty acid oxidation in the liver.

When we focused our attention on the local HSCs-effects in the context of CCl_4-induced liver fibrosis, we found that in contrast to the hepatocyte-specific overexpression model, the HSC overexpression was protective against the development of fibrosis. This suggests that the local adiponectin within the HSCs, not the local concentration from the surrounding hepatocytes, effectively clamps the HSCs in their quiescent state. While we do not know whether adiponectin receptors are present in HSCs or not, we argue that the protective effect is cell autonomous and not dependent on circulating adiponectin. In other words, it is the process of adiponectin production and the accumulation of intracellular adiponectin within the HSCs, not the uptake of adiponectin, which promotes the anti-fibrotic properties in this system. The specific mechanisms, downstream targets, and the function of intracellular adiponectin are still to be clarified.

In summary, our animal model described here offers an inducible system to dissect the role of adiponectin in the HSCs. The use of GFAP promoter is complicated by the fact that it is active in multiple different cell types, and we have to use caution when interpreting the phenotypes. However, a direct comparison to another local hepatic overexpression system from hepatocytes allows us to interpret the results with some level of certainty. The GFAP promoter was the best available promoter we could find at the time these experiments were initiated.

With a LRAT promoter driven rtTA mouse under development in our laboratory, we will be able to assess the systemic metabolic profile of HSCs overexpression in the near future much more carefully. Nonetheless, we see a very strong protective effect of the GFAP promoter driven adiponectin overexpression against CCl_4 induced liver fibrosis. By comparing the results with our previous hepatocyte-specific overexpression models, we hypothesized that local adiponectin treatment in HSCs can stabilized HSCs in the quiescent state and prevent the initiation of fibrogenesis and the subsequent development of steatohepatitis.

Comments

Background

One out of every ten Americans is affected by liver disease. Hepatic steatosis is considered a risk factor for advanced liver disease; however, only a fraction of the affected individuals continue to develop steatohepatitis. Steatohepatitis is characterized by immune infiltration and hepatocyte apoptosis. Depending on the degree of injury, fibrogenesis may be initiated. Thus far, numerous groups have focused their attention on reversing the fibrotic condition. Here, we offer the potential of using adiponectin in local HSCs as a preventative measurement for the further development of fibrosis from simple steatosis.

Research frontiers

Adiponectin, an adipocyte-specific secretory protein, has potent insulin sensitizing effects and affects glucose and fatty acid metabolism on peripheral tissues. Adiponectin is also found to exert anti-inflammatory and anti-fibrogenic effects in cardiovascular and gastrointestinal diseases. The potential of using adiponectin in treating fibrosis has gained considerable attention.

Innovation and breakthroughs

Most of the studies examining the therapeutic potential of adiponectin have been done using systemic adiponectin overexpression, viral delivery, and recombinant protein. Here, our inducible model allows us to overexpress full-length adiponectin at any desired pathological stage and at specific cell type under the control of a cell type specific promoter. This animal model gives us the ability to study and dissect the local effect of adiponectin at specific target cells from the cell autonomous effect.

Application

This study suggests that localized adiponectin treatment in the HSCs can be used as a preventative measure in individuals who are at higher risk for the development of steatohepatitis.

Terminology

Fibrogenesis is a wound-healing, scar forming process in response to injury. Prolonged fibrogenesis leads to accumulation of the scarring tissue, fibrosis can alter liver function and decrease patients' quality of life

Peer Review

This paper examined the effect of localized adiponectin treatment in two different target cells, which resided close to one another. The results are interesting in that only the HSCs-localized adiponectin overexpression can prevent the development of liver fibrosis, whereas the opposite is observed in hepatocytes overexpression.

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Figures

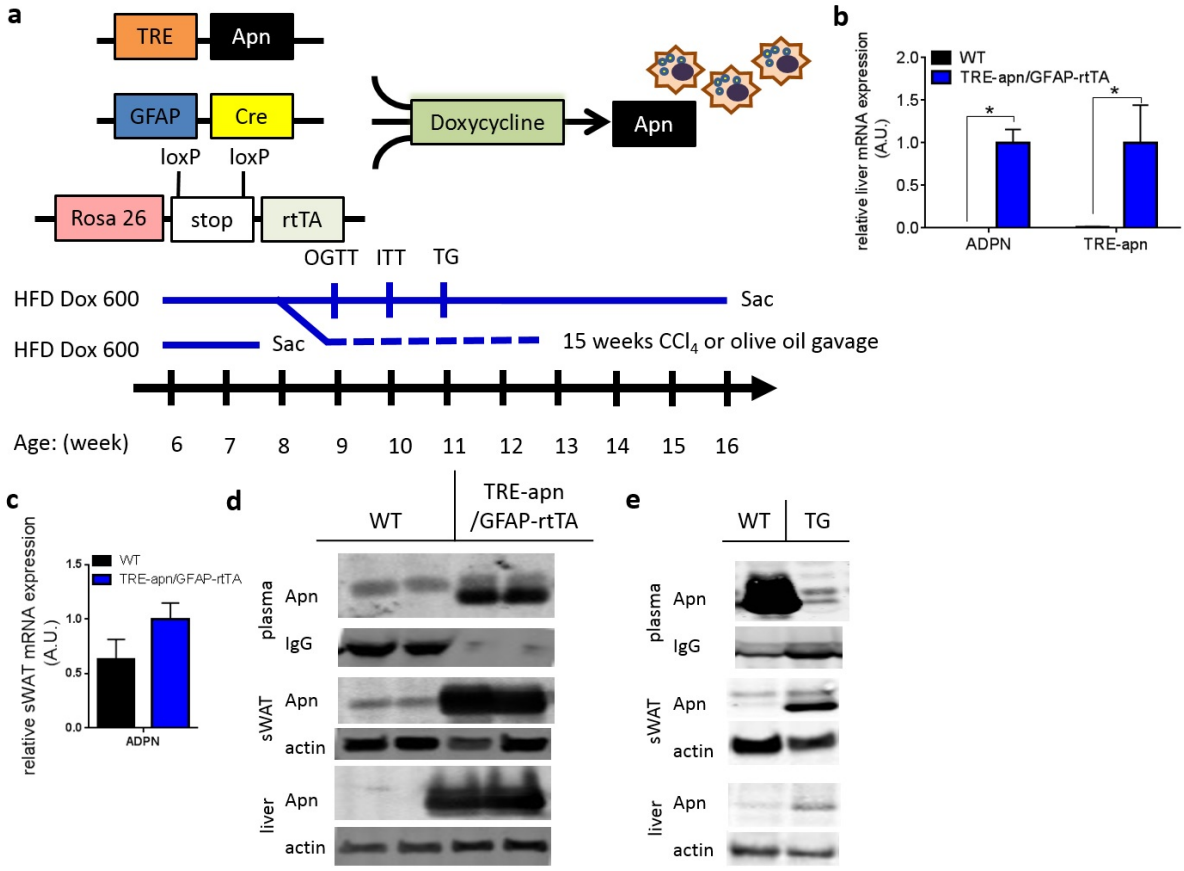

Figure 1: Overexpression of adiponectin from HSCs

a) Schematic diagram of HSCs specific overexpression using GFAP promoter timeline of Dox treatment and metabolic tests for TRE-apn/GFAP-rtTA animals.
b) qPCR analysis of endogenous and exogenous adiponectin mRNA expression in the liver normalized to TRE-apn/GFAP-rtTA after 10 days Dox induction.
c) qPCR analysis of endogenous adiponectin mRNA expression in the subcutaneous white adipose tissue (sWAT) after 10 days Dox induction.
d) Adiponectin western blot from plasma, sWAT, and liver in WT and TRE-apn/GFAP-rtTA after 10 days HFD Dox challenge.
e) Adiponectin western blot from plasma, sWAT, and liver in WT and TRE-apn/GFAP-rtTA mice collected after 10 days HFD Dox challenge and perfusion prior to collection.

*p<0.0 5 compared to WT.Data are presented as means ± SEM, n=3

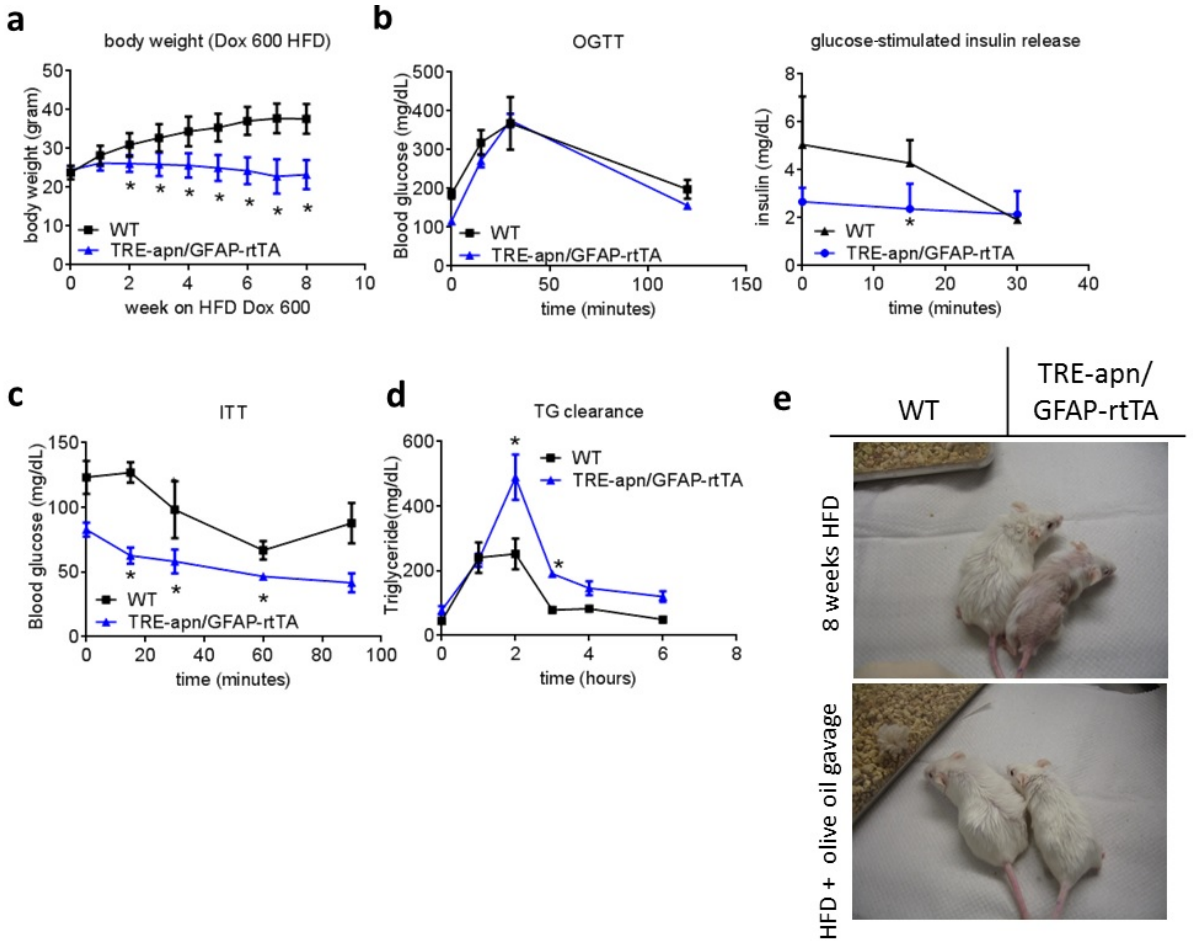

Figure 2: HSCs overexpression alters glucose and lipid metabolism

a) body weight curve in WT and TRE-apn/GFAP-tTA mice during 8 weeks HFD Dox 600 (n=6-10).
b) oral glucose tolerance test (2.5 mg/g BW) and glucose stimulated insulin release during the first 30 minutes of OGTT.
c) insulin tolerance test (0.75 mU/g BW).
d) triglyceride clearance test (15 ml/kg BW of 20% intralipid) (n=4-8).
e) representative animals of WT and TRE-apn/GFAP-rtTA after 8 weeks of HFD-Dox treatment with or without receiving extra olive oil gavage every other day. *$p<0.05$ compared to WT. Data are presented as means ± SEM

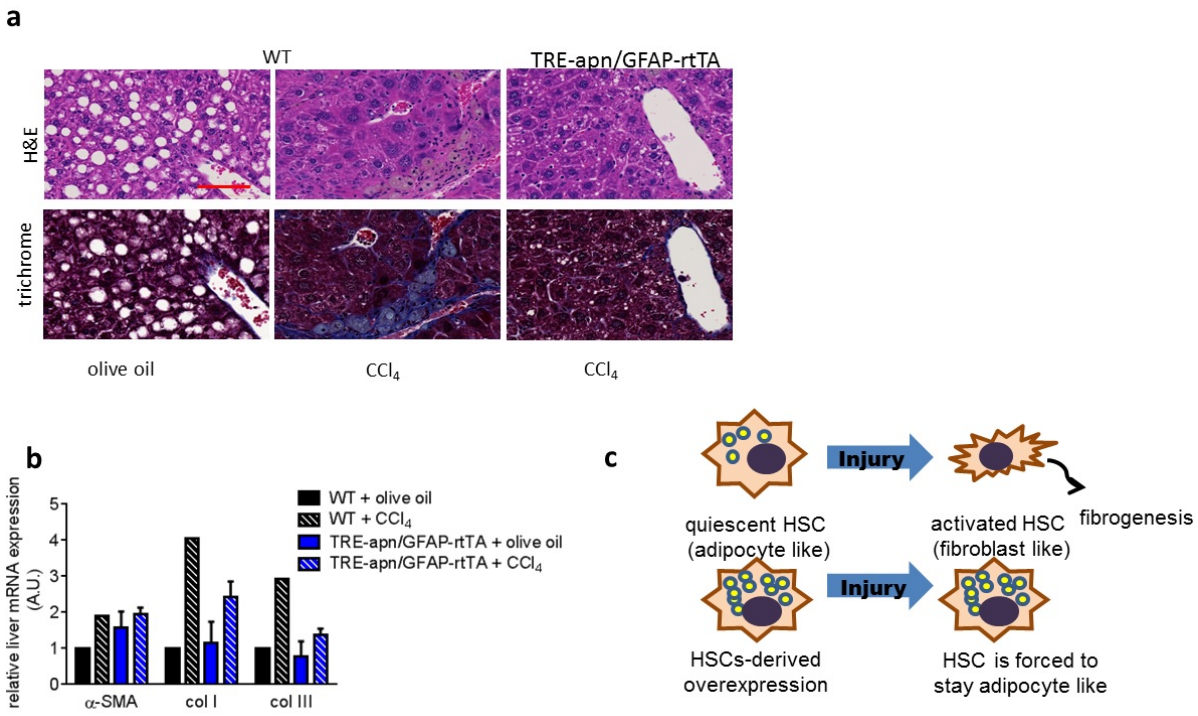

Figure 3: Adiponectin HSCs overexpression protects against CCl_4 -induced liver fibrosis

a) H&E (top) and trichrome stain (bottom) of WT and TRE-apn/GFAP-rtTA liver. Animals received either 40% CCl_4 or olive oil (vehicle) through oral gavage twice a week for 15 weeks following two weeks of HFD Dox treatment. (magnification:10x scale bar 100 μm).

b) qPCR analysis of mRNA expression in liver isolated from either olive oil or CCl_4 treated WT or TRE-apn/GFAP-rtTA animal normalized to WT treated with olive oil (n=3).

c) schematic model of the protective effect of HSC-derived adiponectin overexpression following CCl_4 treatment. The HSC is forced to stay in the quiescent state by the excess adiponectin accumulation within the HSC.

*$p<0.05$ compared to WT. Data are presented as means ± SEM

Supplemental Table

Supplemental table 1: Primer sets for qPCR analysis

Gene	Forward primer	Reverse primer
α-SMA	ACTGGGACGACATGGAAAAG	CATCTCCAGAGTCCAGCACA
ADPN	GTTGCAAGCTCTCCTGTTCC	ATCCAACCTGCACAAGTTCC
β-actin	TACCACAGGCATTGTGATGG	TTTGATGTCACGCACGATTT
collagen I	GAGCGGAGAGTACTGGATCG	TACTCGAACGGGAATCCATC
collagen III	TGGTCCTCAGGGTGTAAAGG	GTCCAGCATCACCTTTTGGT
TRE-apn	GGGGACCACAATGGACTCTA	TTTGCCCCCTCCATATAACA

CHAPTER THREE

THE ROLE OF ADIPOCYTE TOLL-LIKE RECEPTOR 4 IN REGULATING GLUCOSE AND LIPID METABOLISM

(The following section is manuscript prepared for submission as original basic research article)

DICHOTOMOUS ROLE OF ADIPOCYTE TOLL-LIKE RECEPTOR 4 ON OBESSITY-INDUCED INSULIN RESISTANCE

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Abstract

Chronic exposure to diets high in saturated fat (HFD) increases the prevalence of obesity and contributes to the development of low-grade inflammation and insulin resistance. One of the mechanisms accounting for the obesity-associated inflammation and insulin resistance is the activation of Toll-like receptor 4 (TLR4). Here, we investigated the specific role of adipocyte TLR4 in mediating the effects of lipids on glucose metabolism through inducible, cell-specific deletion of TLR4 in adult mice (we refer to these mice as "Tadipo mice"). After chronic HFD-feeding, elimination of TLR4 in adipocytes resulted in reduced TLR4 expression in peritoneal macrophages and the liver. Consistent with our previous observations arguing for an important role of adipocyte inflammation in healthy adipose tissue expansion and remodeling, Tadipo mice exhibit mildly reduced insulin sensitivity and showed reduced lipid clearance. However, when subjected to an acute challenge with lard oil infusion during a euglycemic clamp study, Tadipo mice maintained higher insulin sensitivity than the paired controls. Our results demonstrate a dichotomous effect of TLR4 on adipose tissue functionality, with a deteriorated phenotype in Tadipo mice during a *chronic* HFD challenge due to the lack of adipose tissue remodeling, and as a mediator of insulin resistance during an acute challenge with saturated fatty acids.

Introduction

Obesity is the most common cause of insulin resistance in the Western population. The increasing prevalence of obesity also correlates with the increasing prevalence of type 2 diabetes. Studies within the past ten years have been heavily focused on identifying the link between obesity and insulin resistance. Chronic low-grade inflammation, one of the hallmarks of obesity, is thought to be a major contributor to obesity-induced insulin resistance (1). The activation of TLR4 signaling and the subsequent release of inflammatory cytokines through regulation of several important transcription factors has been recognized as a critical player in connecting obesity-associated inflammation and insulin resistance (2).

TLR4 was first identified as an endotoxin receptor. It is a pattern-recognition receptor that functions to recognize molecules shared among pathogens to initiate host defense innate immune response (3). A number of studies using TLR4 deficient mice revealed attenuated diet-induced inflammation and insulin resistance (4-6). However, the specific cell type involved in this process is unclear, since TLR4 is widely expressed throughout the body, and the net phenotype observed may be a reflection of a composite response that many different cell types contribute to differentially. Our groups have previously demonstrated the role of hepatocyte and macrophage-specific null mice for TLR4 in mediating obesity-induced inflammation, insulin resistance and hepatic steatosis (7). Here we investigate the role of adipocyte TLR4 by using an adipocyte specific inducible knockout system.

In differentiated 3T3-L1 adipocytes, LPS treatment can stimulate IL6 production, demonstrating the classic cellular endotoxin receptor response of TLR4 *in vitro*. (8). Later, Shi et al. demonstrated that free fatty acids (FFAs) can also induce the release of pro-inflammatory cytokines via TLR4 signaling using both a RAW264.7 macrophage cell line and TLR4 deficient mice (9). Although FFAs do not bind to TLR4 directly, FFAs can induce inflammatory responses in a TLR4 dependent manner (10). One possible mechanism linking TLR4 activation with insulin resistance is through ceramide biosynthesis. Activation of TLR4 by FFAs can stimulate ceramide production, which in turn inhibit Akt and contribute to the development of insulin resistance (11).

Back in 2000, we reported that TLR4 is highly expressed on the cell surface of adipocytes (8). It is well recognized that chronic low-grade inflammation associated with prolonged over feeding can contribute to the development of insulin resistance. We previously demonstrated that adipocytes can exert potent pro-inflammatory effects on local macrophages. This then results in an enhanced secretion of cytokines and can ultimately lead to an elevation in systemic cytokine levels in the obese state (12). We hypothesized that adipocyte TLR4 is a key regulator of obesity-associated inflammatory responses, and that the adipocyte TLR4 can contribute to the development of insulin resistance through up regulation of cytokines and also lead to the activation of ceramide biosynthesis.

Results

Generation of adipocyte-specific TLR4 deletion ("Tadipo mice")

We took the same animals that we employed for the hepatocyte-specific knockout and combined them with the doxycycline (Dox)-dependent inducible system to create adipocyte-specific TLR4 null animals. Upon binding to Dox, the adiponectin promoter driven cre recombinase will excise exon 2 and exon 3 of *TLR4* from mature *adipo*cytes, giving rise to our Tadipo mice (**Fig. 1a**). Our adiponectin promoter is only activated in mature adipocytes (13). Therefore, we can avoid any potential phenotype due to developmental effects. Tadipo mice are still LPS responsive since this deletion is highly selective. After HFD exposure, we can observe about 60~80% reduction in the TLR4 mRNA in the adipocyte fraction (f. f.) isolated from the subcutaneous and gonadal white adipose tissues (sWAT and gWAT). Interesting, we also observed a decrease in TLR4 mRNA in the peritoneal macrophages and the liver without any local gene rearrangements within these cells, i.e. macrophages and hepatocytes down-regulate TLR4 as a response to the lack of TLR4 signaling in adipocytes (**Fig. 1b-c**). Additionally, this reduction in TLR4 mRNA was not seen when the Tadipo mice were fed a chow diet containing Dox (**Suppl. Fig. 1a**). Multiple pro-inflammatory markers, such as IL1β, were also significantly reduced in the adipocyte fractions, peritoneal macrophages and in the liver (**Fig. 1d**). This reduction in TLR4 expression in the peripheral tissues is a purely transcriptional regulation in response to the adipocyte TLR4 deletion, suggesting a crosstalk event between the adipocyte, peritoneal macrophages and the liver.

Tadipo mice display decreased WAT macrophage infiltration, a slightly decreased insulin sensitivity, decreased systemic adiponectin expression and redistribution of WAT

During the 10 weeks of HFD challenge, there was no difference in body weight between the Tadipo mice and littermate controls lacking cre (TLR4 $^{fl/fl}$) (**Fig. 2a**). However, we can observe a clear reduction in white adipose tissue macrophage infiltration judging by reduced crown-like structures in both the sWAT and gWAT after HFD treatment (**Fig. 2b**). Despite the clear reduction in the inflammatory response in white adipose tissue, we do not observe any differences during an oral glucose tolerance test (OGTT); the decreased in insulin

sensitivity during an insulin tolerance test (ITT) is very marginal (**Fig. 2c-d**). Interestingly, we observed significant reduction in both circulating and intracellular adiponectin level after chronic HFD exposure (8~10 weeks) (**Fig. 2e**). When we dissected and weighted each of the fat pads at the end of the HFD challenge, we noticed the Tadipo mice had increased sWAT and decreased gWAT compared to control animals (**Fig. 2f**).

Adipocyte TLR4 deletion leads to increased steatosis and decreased insulin sensitivity after chronic HFD exposure

Increased lipid deposition was observed in both the brown adipose tissue (BAT) and the liver after chronic HFD exposure (8~10 weeks) (**Fig. 3a**). This increase in hepatic steatosis was accompanied by increased expression of fatty acid synthesis-related genes in the liver (**Fig. 3b**). While we do not question the relevance of chronic systemic inflammation, we argued in the past that a reduction of inflammation in the local environment can lead to detrimental effects (14). Previously, we demonstrated that local adipose tissue inflammation is critical to maintain healthy adipose tissue expansion and remodeling in response to HFD exposure using several mouse models. Given the observations in the Tadipo mice, we observe a decreased macrophage infiltration, decreased insulin sensitivity and a redistribution of WAT. This suggests that that the Tadipo mouse may be another model of insufficient local adipose inflammation. To define this issue further, we performed hyperinsulinemic euglycemic clamps. Indeed, upon chronic HFD exposure (5 weeks HFD, BW=30g), Tadipo mice display reduced insulin sensitive and reduced glucose uptake during a hyperinsulinemic euglycemic clamp study (**Fig. 3c-e**).

Tadipo mice display increased insulin sensitivity during hyperinsulinemic euglycemic clamps with acute lard infusion

The increased lipid deposition in BAT and the liver observed in earlier sections suggested to us that deleting TLR4 from adipocytes might affect lipid metabolism. We orally gavaged Tadipo mice and control animals with intralipid to assess the lipid clearance (**Fig. 4a**). The Tadipo mice display an enhanced lipid excursion; confirming that deletion of adipocyte

TLR4 can lead to impairment in lipid clearance. We were interested to find out if Tadipo mice respond differently to chronic versus acute lipid exposure. Given that after chronic HFD exposure, Tadipo mice show reduced insulin sensitivity compared to control animals (**Fig. 3c-e**), we repeated the experiment when the mice were treated with HFD for three weeks when the mice had not yet gained a significant amount of weight (BW=25g). With the shorter time course of HFD exposure, we found that the Tadipo mice were more insulin sensitive and more efficient at glucose clearance compared to littermate controls (**Fig. 4b-d**). To further simulate the effect of acute lipid exposure, we pre-infused the chronic HFD challenged animals (5 weeks HFD, BW=30g) with lard oil emulsion prior to standard clamp studies on clamp day. Surprisingly, Tadipo mice were significantly more insulin sensitive and efficient at glucose clearance compared to littermate controls (**Fig. 4e-g**). During the lipid infusion clamps, lower insulin levels were found in Tadipo mice during both basal and steady state, suggesting that the effect of insulin sensitivity was greater than we observed in the glucose infusion rates (**Suppl. Fig 2**). We repeated the acute lipid infusion clamp studies with animals on chow diet (7 days Dox-containing chow diet fed) and found the same pattern as observed under chronic HFD condition. In fact, under chow conditions, Tadipo mice receiving lard oil infusions have even higher glucose infusion rates compared to Tadipo mice receiving control infusions (**Suppl. Fig. 1g-h**).

Discussion

When we first examined the Tadipo mice, there was no obvious systemic metabolic phenotype despite the significantly decreased inflammatory response in white adipose tissue after HFD. In fact, the impact of the hepatocyte-specific TLR4 deletion seemed more severe, such that we observed an effect in adipose tissue secondary to the hepatocyte-specific deletion after the HFD challenge (7). Here, working with the adipocyte-specific deletion, we show further evidence for the strong crosstalk between adipocytes, peritoneal macrophages and liver during the development of obesity. We repeated the metabolic characterization and gene expression analysis of the Tadipo mice under chow-fed conditions and the mice were essentially indistinguishable from wildtype, confirming that the phenotypic changes are specific to HFD exposure. We do not know whether the reduction in TLR4 expression can be observed in resident macrophages in organs different from liver and the peritoneum. Since the Tadipo mice remain LPS responsive, we speculate that the reduction in macrophage TLR4 is not global but more restricted to macrophages directly related to adipose tissue physiology.

Similar to our previously described adipocyte-specific anti-inflammation models, Tadipo mice develop hepatic steatosis and a deteriorated metabolic phenotype after a chronic HFD challenge. Keeping in mind that TLR4 may also serve as a link between obesity and lipid-induced insulin resistance through ceramide biosynthesis, we evaluated the effect of adipocyte TLR4 in lipid metabolism. β3 agonist stimulated white adipose tissue lipolysis is not affected by the adipocyte TLR4 deletion (*data not shown*). However, the Tadipo mice do have a slower lipid clearance compared to control animals after intralipid gavage.

In the event of an acute lipid exposure, deleting TLR4 from adipocytes is advantageous to the overall system. Deleting TLR4 can ablate the downstream inhibitory effects on Akt signaling by preventing FFA-mediated TLR4 activation. As a result, insulin sensitivity is improved, and that is quite apparent upon clamping the mice after an acute challenge with lard oil infusion, both in a HFD and a chow-fed background. In contrast, during chronic HFD exposure, deleting TLR4 from adipocytes is disadvantageous to the system. Despite the remarkable redistribution of white adipose tissue towards a more favorable distribution with enhanced sWAT, the significant reduction in systemic adiponectin can be viewed as a sign of

unhealthy adipose tissue. Since local adipose inflammation is essential to appropriately adapt the tissue for expansion, the lack of TLR4 mediated inflammatory response leads to a reduced capacity to remodel, impaired adipose tissue expansion, and ultimately decreased insulin sensitivity.

When we deleted TLR4 from adipocytes, we observed a dichotomous response, with beneficial and disadvantageous results that affect both adipocytes and peripheral tissues. We also observed additional temporal differences in response to lipid, with differential responses to acute versus chronic exposure. The combination of all of the above responses averages out to a relatively mild overall phenotype. Nevertheless, the complexity of the metabolic effects also hints at the importance of TLR4 in adipocytes. Our studies further corroborate our previous findings regarding the local inflammatory response in the maintenance of tissue architecture. Since we have performed loss of function studies here, it will be of equal interest to look at cell-specific reactivation studies of TLR4 in an otherwise TLR4 null background. Our groups are using two separate approaches to do this currently and we hope to further validate our working model to better understand the role of selective, adipose tissue-specific inflammation in the context of obesity-induced insulin resistance.

Author Contributions

C.T., J.K.E. and P.E.S. designed the experiments and wrote the manuscript. L.J., K.S., and Y.K. generated and mouse models, X.L., J.A.J., and W.L.H. assisted with clamp study, C.T., Q.A.W., performed animal experiments and analyzed data.

Acknowledgements

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Methods

Mice

Mice were maintained on a 12 hour dark/light cycle and housed in groups of three to five with unlimited access to water, chow (No. 5058, Lab-Diet) or high fat (60% calories from fat) doxycycline containing diet (600mg kg^{-1}) (HFD Dox)(S4107, Bio-Serv) as indicated for the individual experiments. The Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center, Dallas, has approved all animal experiments. The use of apnP-rtTA and the TRE-cre mice have been previously describe (13).

Hyperinsulinemic euglycemic clamp study

Surgery and experimental set up were similar to previous experiments (11,15). Briefly, body weight and age matched animals were fast for three hours prior to 90 minutes infusion with [^{3}H]-glucose (5 μCi bolus followed by 0.05 μCi/min) to measure glucose turnover, blood samples from the cut tail were taken for measurements of basal insulin and glucose as well as to calculate glucose turnover. The clamp was started at 0 min with a continuous insulin infusion (10mU/kg/min: Humulin, Eli Lilly), and the [^{3}H]-glucose was increased to 0.1 μCi/ min to minimize changes in specific activity. A variable glucose infusion rate (50% dextrose) was used to maintain blood glucose at 150 mg/dl. Blood samples were taken every 10 min during the steady-state period (80–120 min) to determine hepatic glucose production and glucose disposal rate. For lipid infusion clamp, the mice fed on Dox containing chow diet, Dox contain HFD for 3 weeks (BW=25 gram) and over 3 weeks (BW=30g) were pre-infused with lard oil emulsion or glycerol for three hours prior to tracer infusion.

Adipocyte and SVF isolation.

sWAT and gWAT were collected from TLR4$^{fl/fl}$ and Tadipo mice fed HFD for 10 weeks. Tissues were minced and digested using collagenase (Gibco, #17703-034) at 37°C for 1.5 h with shaking. The cell suspension was filtered through a 250 mm mesh, washed with DMEM containing 10% FBS, and centrifuged at 800g for 1min at room temperature. The floating adipocytes were collected, centrifuged at 500g for 1min and dissolved in Trizol for RNA isolation. The remaining cell pellets containing SVF were put on ice immediately. The pellets

were passed through 40 mm cell strainer, washed with cold DMEM buffer and centrifuged at 500g for 5min at 4°C. Then, SVF were collected as pellets and resuspended in Trizol for RNA isolation

Western blot analysis

Adipose tissue samples were homogenized on ice in T-PER (Thermo Scientific, USA) supplemented with protease inhibitor cocktail (*cOmplete* mini, EDTA-free, Roche). This was followed by low speed centrifugation (3,000xg at 4°C), in order to remove the fat cake from the top. The tissue extracts were cleared at 15,000 x g for 20 minutes at 4°C and total protein concentration measured with BCA Protein Assay Kit (Pierce Protein Research Products, Thermo Scientific, USA). Equal amounts of protein from adipose tissue were mixed with 5x Laemmli sample buffer with DTT and boil at 95°C for 5 minutes. The samples were resolved on 4-15% TGX gels (Bio-Rad, USA), followed by semi-dry transfer to PVDF membrane (Bio-Rad, USA). Blots were probed with rabbit polyclonal anti mouse adiponectin antibody(16). Bound antibodies were detected with IRDye800-conjugated anti-rabbit secondary antibodies (Rockland). Membranes were scanned with the LI-COR Odyssey Infrared Imaging System. Actin controls were visualized with an anti-mouse actin monoclonal antibody from Cell Signaling with IRDye680-conjugated anti-mouse secondary antibodies (Rockland).

Blood biochemistry

Insulin levels were measured by commercial ELISA kits from Millipore (Billerica, MA, USA). Glucose levels were determined with Sigma Diagnostics Glucose Reagents (Sigma, Sigma Aldrich, USA), Triglycerides were measured using Infinity Triglycerides Reagent (Thermo Scientific, USA) and free fatty acids levels were measured with NEFA-HR(2) (Wako Pure Chemical Industries, Japan).

Oral glucose and insulin tolerance tests

Oral glucose tolerance test (OGTT) was performed in mice on HFD Dox for 3 weeks starting at 6 weeks of age. One week later, the same mice were used for insulin tolerance test (ITT). For OGTT, mice were fasted for 4 hour during light phase and blood samples were drawn from tail vein before and 15, 30, 60, and 120 minutes after oral gavage with 2.5 mg/g BW of glucose in

PBS. For ITT, 4-h fasted mice were injected intraperitoneally with recombinant human insulin (Novo Nordisk, Inc.) at 0.75 mU/g BW and blood samples were collected before and 15, 30, 60, and 90 minutes after.

Triglycerides clearance

Triglycerides clearance (TG clearance) was performed in mice on HFD Dox for 5 week starting at 6 weeks of age. The mice were fasted for 6 hour during light phase and blood samples were drawn from tail vein before and 1, 2,3,4,6 hours after oral gavage with 15ml/kg BW of 20% intralipid (Fresenius Kabi Clyton, L.P.).

Quantitative real-time RT-PCR

Tissues were snap freeze in liquid nitrogen and stored at -80°C. Trizol Reagent (Invitrogen, USA) extraction followed by RNA purification using RNeasy Mini Kit and RNase-Free DNase (Qiagen, USA). RNA was reverse transcribed to cDNA by iScript cDNA synthesis kit (Bio-Rad, USA) and SYBR Green (Applied Biosystems, life technologies, USA) was used for the quantitative PCR reactions. The relative expression level was calculated by the comparative Ct method using β-actin as endogenous control. Primer sequences can be found in **Supplemental Table 1**.

Statistical Analysis

All the results are presented as means ± Standard Error of the Mean (SEM). Differences between two groups were determined for statistical significance by a standard two-tailed Student t test. Significance was accepted at a value of $p < 0.05$

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Figures

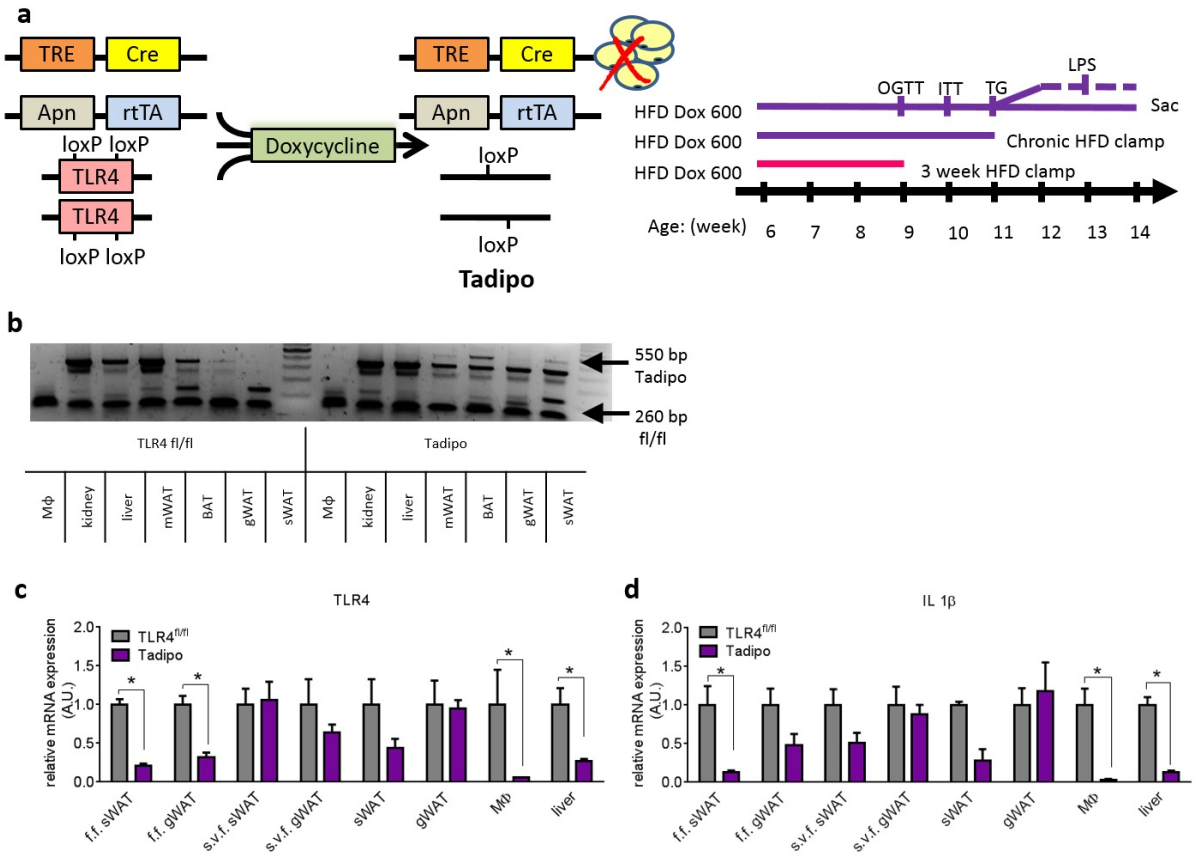


Figure 1: Generation of adipocyte-specific TLR4 deletion (Tadipo mice)

a) schematic model of doxycycline inducible adipose tissue specific deletion (Tadipo mice) timeline of HFD Dox treatment and metabolic tests.

b) gene rearrangment in various tissues isolated from 8 weeks HFD Dox challenged Tadipo mice.

c-d) qPCR analysis of TLR4 and IL1 β mRNA expression in various tissue normalized to TLR4 $^{fl/fll}$ after 8 weeks HFD Dox challenge.

*p<0.0 5 compared to WT.Data are presented as means ± SEM. n=4

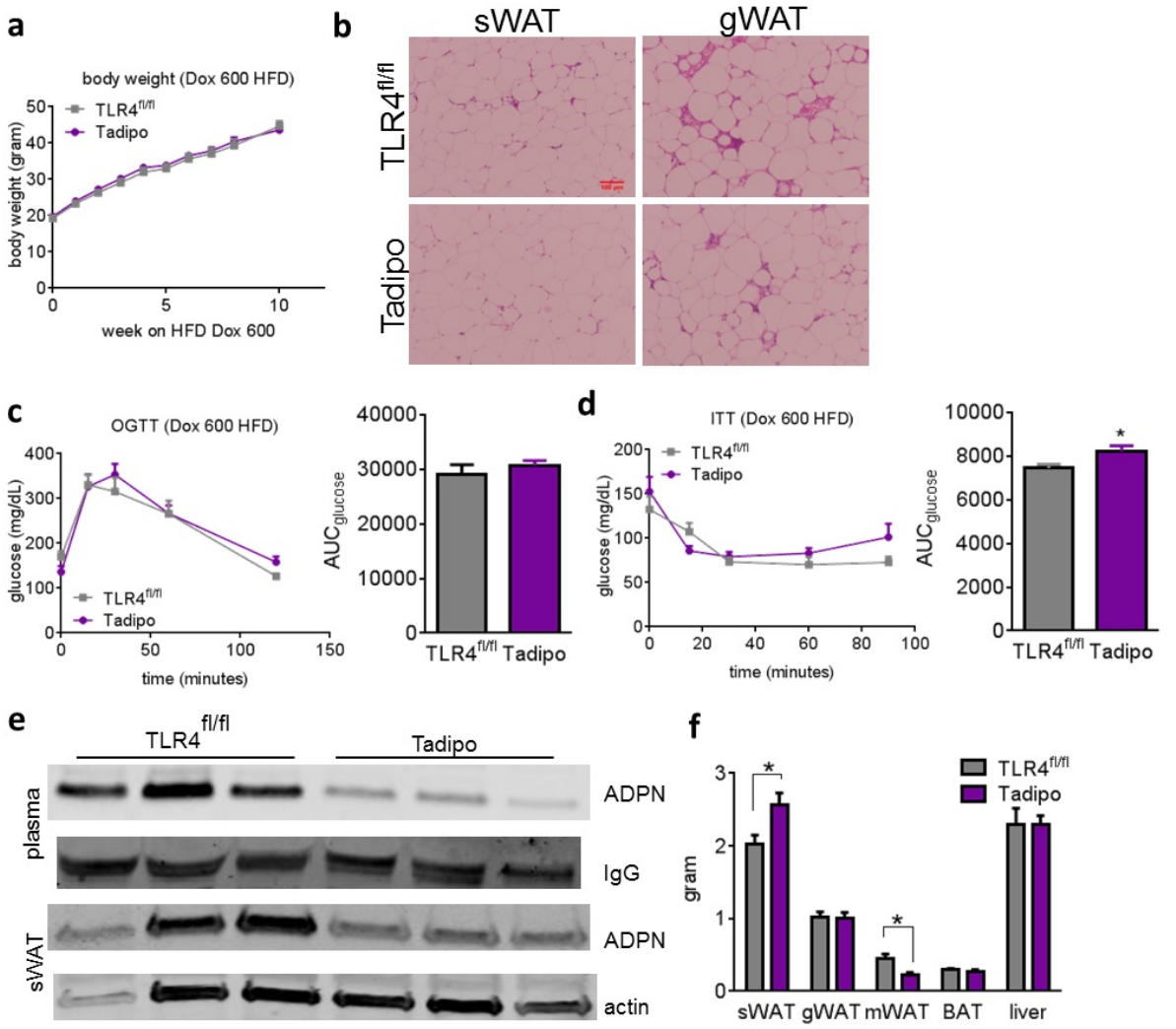


Figure 2: Tadipo mice display decreased WAT macrophage infiltration, a slight decrease in insulin sensitivity, and decreased systemic adiponectin expression

a) 10 week body weight follow up during HFD challenge (n=10~15).

b) H&E staining of subcutaneous and gonadal white adipose tissue (sWAT and gWAT) after 8 weeks HFD challege.

c) oral glucose tolerance test (2.5 mg/g BW).

d) insulin tolerance test (0.75 mU/g BW) (n=6~8).

e) Western blot of adiponectin in plasma and the sWAT isolated from Tadipo and control animals 8 weeks post HFD challenge. Relative intensity is normalized to either IgG or actin signal.

f) tissue weight after 8 weeks HFD challenge.

*p<0.05 compared to TLR4 $^{fl/fll}$. Data are presented as means ± SEM

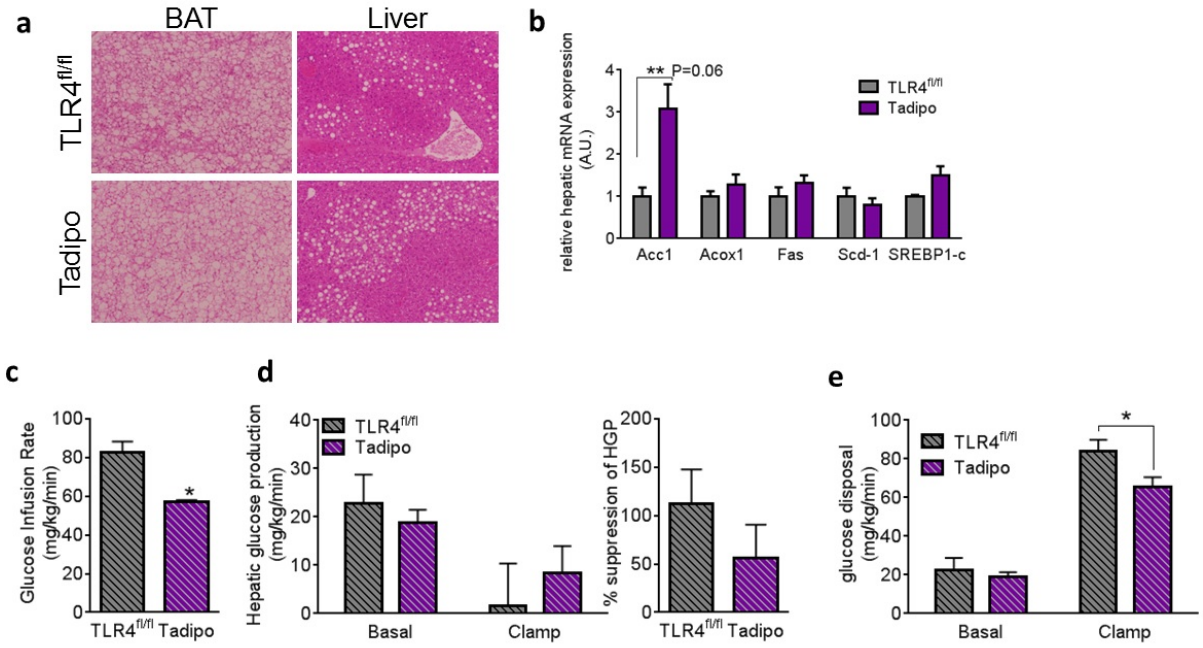


Figure 3: Adipocyte TLR4 deletion leads to increased steatosis and decreased insulin sensitivity after chronic HFD exposure

a)H&E staining of brown adipose tissue (BAT) and liver after 8 weeks HFD challenge.
b) lipogenesis-related gene expression in the liver after 8 weeks HFD challenge.
c-e) glucose infusion rate (c). hepatic glucose production and suppression of hepatic glucose production (d) and glucose disposal (e) during hyperinsulinemic euglycemic clamp during hyperinsulinemic euglycemic clamp after chronic HFD feeding (chronic HFD clamp, 5 weeks HD, BW=30) (n=4).
*P<0.05 compared to TLR4 $^{fl/fl}$. Data are presented as means ± SEM.

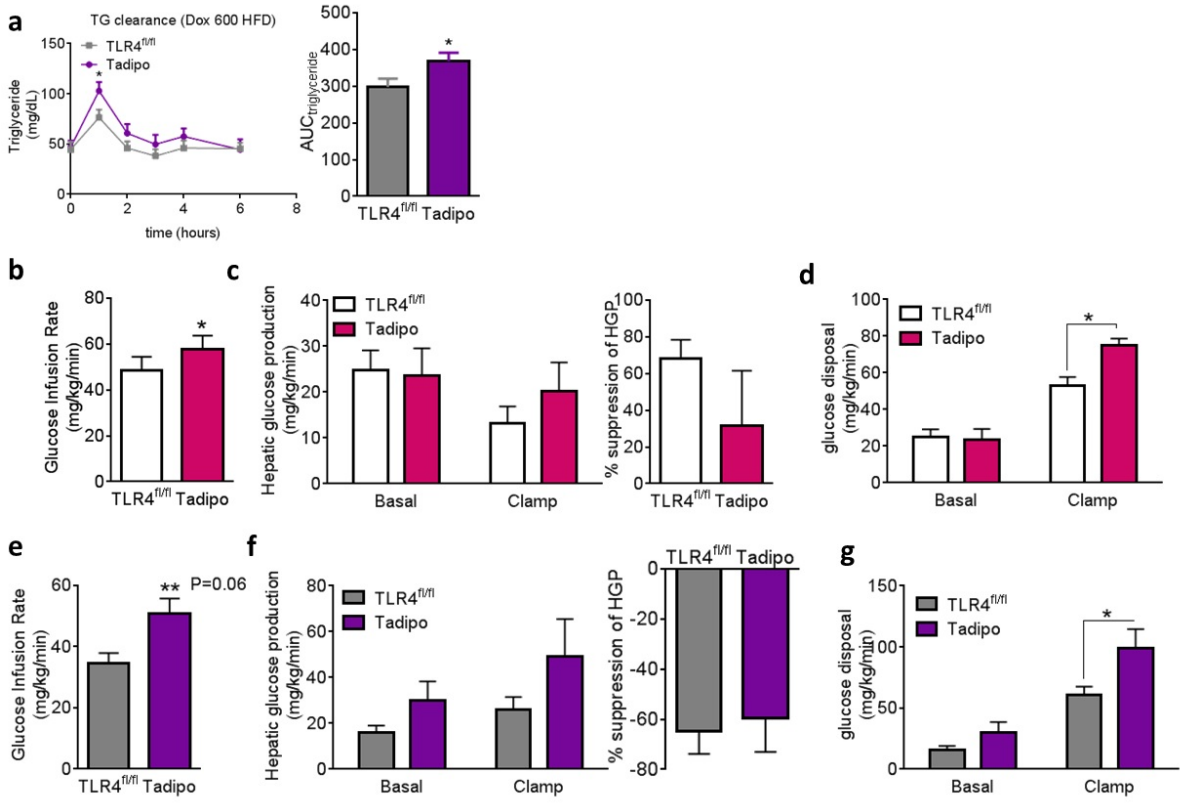


Figure 4: Tadipo mice display increased insulin sensitivity during hyperinsulinemic euglycemic clamp upon acute lard infusion after chronic HFD exposure

a) triglyceride clearance test (15ml/kg BW of 20% intralipid) (n=6~8) after 5 weeks HFD treatment.

b-d) glucose infusion rate (b).hepatic glucose production and suppression of hepatic glucose production (c), and glucose disposal (d) during hyperinsulinemic euglycemic clamp after 3 week HFD feeding (BW=25g, n=8~10).

e-g) glucose infusion rate (e), hepatic glucose production and suppression of hepatic glucose production (f), and glucose disposal (g) during hyperinsulinemic euglycemic clamp with lard infusion after chronic HFD feeding (chronic HFD clamp, 5 weeks HD, BW=30) (n=4~5).

*P<0.05 compared to TLR4 $^{fl/fl}$. Data are presented as means ± SEM

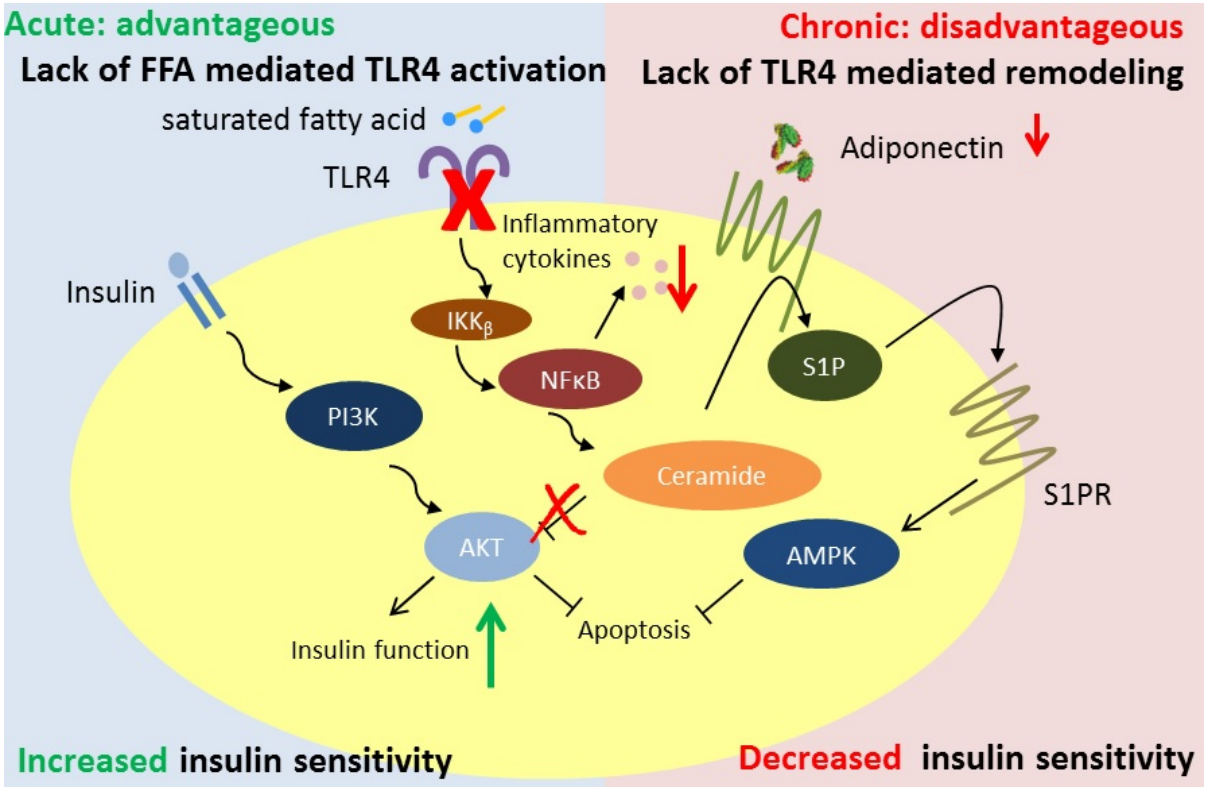
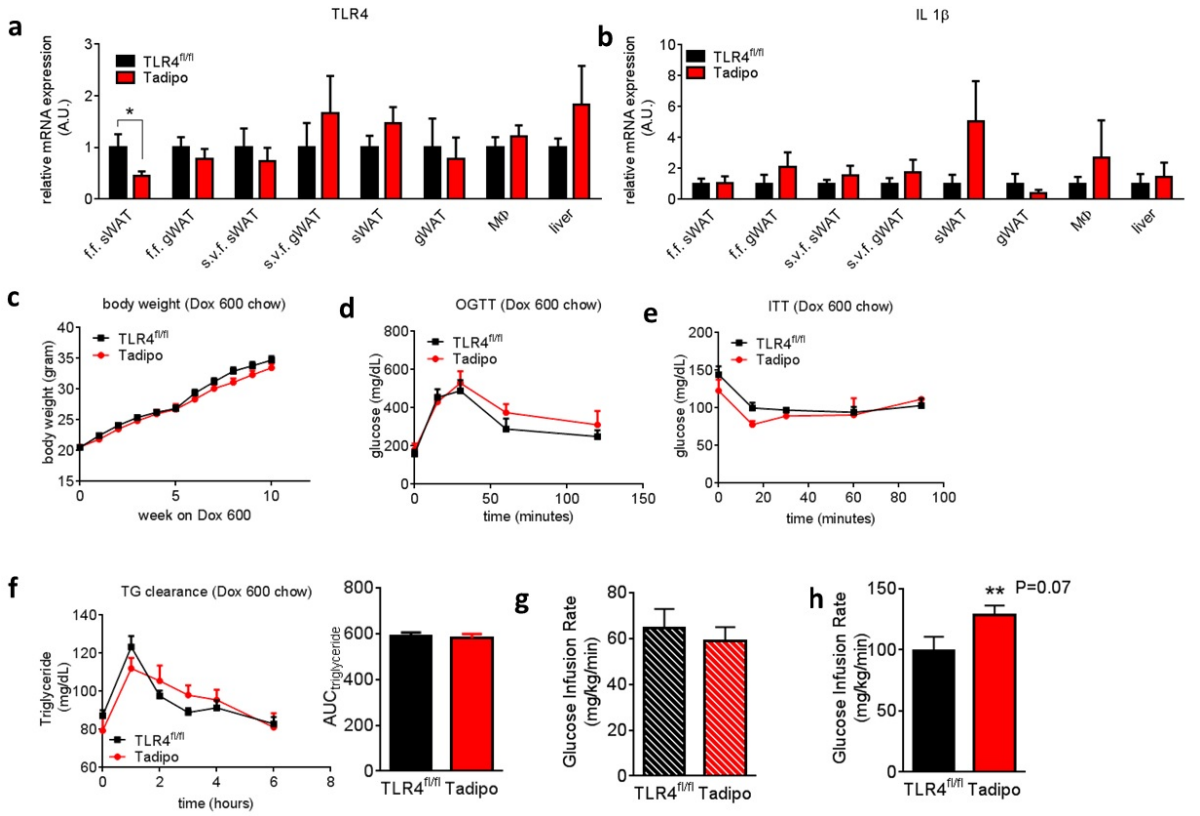


Figure 5: Working model of the effects of adipocyte-specific TLR4 deletion

In the acute lipid exposure, lack of adipocyte TLR4 is advantageous and can increase insulin sensitivity due to lack of FFA mediated TLR4 activation. However, during chronic HFD exposure, lack of adipocyte TLR4 is disadvantageous and result in decreased insulin sensitivity due to lack of TLR4 mediated adipose remodeling and healthy expansion.



Supplemental Figure 1: Characterization of Tadipo mice on chow diet

a-b) qPCR analysis of TLR4 and IL1 β mRNA expression in various tissue normalized to $TLR4^{fl/fll}$ after 8 weeks Dox containing chow diet induction

c) 10 week body weight follow up on chow diet (n=10~15).

d) oral glucose tolerance test (2.5 mg/g BW).

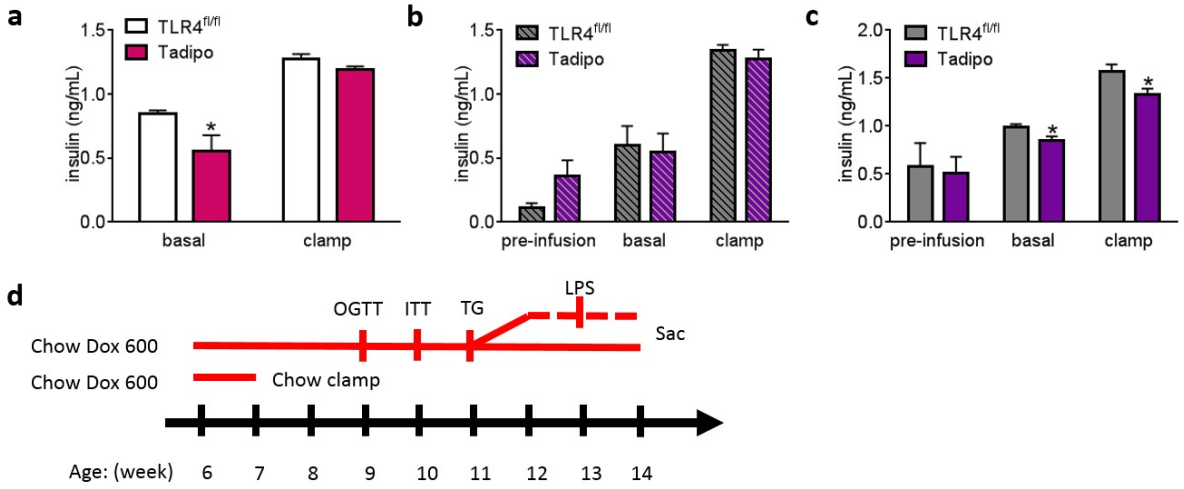
e) insulin tolerance test (0.75 mU/g BW).

f) triglyceride clearance test (15ml/kg BW of 20% intralipid) (n=4).

g) glucose infusion rate during hyperinsulinemic euglycemic clamp after 7 days Dox induction (n=4~5).

h) glucose infusion rate after acute lard infusion during hyperinsulimic euglycemic clamp after 7 days induction with Dox containing chow diet (n=4~5).

*p<0.05 compared to $TLR4^{fl/fll}$. Data are presented as means ± SEM



Supplemental Figure 2: Insulin levels during hyperinsulinemic euglycemic clamp

a-c) plasma insulin measured at basal and steady state during during hyperinsulinemic euglycemic clamp after 3 week HFD, chronic HFD, and lard infusion.

d) timeline of dox containing chow induction and metabolic tests.

*p<0.05 compared to TLR4 $^{fl/fll}$. Data are presented as means ± SEM

Supplemental Tables

Supplemental Table 1: Primer sets for qPCR analysis

Gene	Forward primer	Reverse primer
Acc1	TGGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
Acox 1	TCCCGATCTGCGCAAGGAGC	CTGGTGAAGCAAGGTGGGCA
β-actin	TACCACAGGCATTGTGATGG	TTTGATGTCACGCACGATTT
Fas	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
IL 1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Scd1	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
SREBP1-c	GGAGCCATGGATTGCACATT	CAGGAAGGCTTCCAGAGAGG
TLR4	GACACCAGGAAGCTTGAATCC	TGATCCATGCATTGGTAGGTAA

Supplemental Table 2: Primer sets for TLR4 deletion screening

Gene	Forward primer	Reverse primer
fl/fl	GACTTGAGAGTTATGTAACACCTG	TCCTATAGACCAGTCTGGCCTTAA
Tadipo	GACTTGAGAGTTATGTAACACCTG	GTGGCTATGTTCCAGTTTGAATG

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTION

Summary

As the prevalence of obesity has increased globally over the last few decades, the quest to identify a magic bullet that can resolve the major obesity-associated comorbidities is a very high priority. One of the hallmarks of unhealthy hypertrophic adipose tissue is the reduction in circulating adiponectin levels. Adiponectin, an adipocyte-specific secretory protein, has been found to exert multiple cell-protective effects on various tissues under numerous metabolic conditions. The pleiotropic effects of adiponectin and the underlying mechanisms therefore render it a promising area in the treatment of obesity-associated metabolic diseases.

In my studies, I first set out to better understand how we can increase the production and release of adiponectin from the adipocyte. By using a series of target cell-specific and doxycycline (Dox)-inducible transgenic animal models, we demonstrated a unique post-transcriptional regulation for adiponectin expression within the adipocyte. Contrary to adipocyte regulation, we identified that local overexpression of adiponectin from hepatocytes and hepatic stellate cells (HSCs) gives rise to increased circulating levels of adiponectin. Despite the different post-translational protein modifications of the liver-derived adiponectin being quite apparent, we can still see adiponectin-mediated improvements in glycemic and lipid control. With the two liver-derived overexpression models at hand, we described the promising nature of local HSC-specific overexpression of adiponectin in the prevention of liver fibrosis.

Obesity-associated inflammation is another hallmark of unhealthy adipose tissue. TLR4, a potential link between the innate inflammatory response and lipid-induced insulin resistance, came to our attention in the context of adipose tissue inflammation. By using a Dox-inducible system of adipocyte-specific deletion of TLR4 (Tadipo mice), we examined the role of adipocyte TLR4 in mediating obesity-associated inflammation and insulin

resistance. Upon deletion of TLR4 from adipocytes, we observed a mix of both beneficial and deteriorated phenotypes, largely affecting both adipocytes and peripheral tissues. More specifically, since a reduction in circulating adiponectin levels was observed in Tadipo mice, this could certainly contribute to some of the metabolic outcomes. However, several other factors could also contribute to the overall metabolic phenotype. Specifically, we also reported a temporal element within the phenotype. A dichotomous response of the adipocyte TLR4 deletion with regards to insulin sensitivity in response to chronic *versus* acute lipid exposure was evident. The combination of all of these metabolic readouts leaves us with forces pulling the system at several different directions. My studies using this adipocyte-specific TLR4 deletion model added further insights into the importance of obesity-associated adipose tissue inflammation in the development of systemic insulin resistance.

Future Directions

The mechanism of the regulation of adiponectin secretion has been an unanswered question for the past twenty years. Although we have not yet fully identified the key mechanisms of this regulatory machinery, the more we invest in this area with the availability of our novel inducible mouse models, the more we appreciate the complexities of the system. How does the adipocyte decide how much adiponectin needs to be secreted? What options does the adipocyte have to take advantage of a feedback loop that allows the remarkable stability of plasma adiponectin levels despite a short turn-over of the protein of less than one hour? Why is this system selectively in place in the adipocyte, however, other cell types can easily overcome this limit and secrete vast amounts of adiponectin? The answers to these questions are at the very core for a better understanding of the physiology of adiponectin. The availability of inducible systems combined with the stark contrast between the limits to overexpression between *in vitro* and *in vivo* conditions offer a new platform for discovery.

Identifying the differences in key protein modifications in between the endogenous form of adiponectin and liver-derived adiponectin could certainly help us in understanding the regulation of adiponectin at the level of the adipocyte. We are currently backcrossing

the hepatocyte-specific overexpression model of adiponectin (TRE-apn/Alb-rtTA mice) into an adiponectin knockout background to harvest pure liver-derived adiponectin from serum, and compare this with endogenous adiponectin to ultimately evaluate the adipocyte-specific modifications biochemically.

The use of the GFAP promoter in our HSCs study added complexity, due to the lack of specificity of the promoter expression. However, it was the most suitable and available promoter we could locate at the time. On a positive note, with our newly generated LRAT promoter-rtTA mouse, we will be able to assess the systemic metabolic profile and further validate the local effect of a HSCs overexpression in the near future.

Bearing in mind the multiple cell-protective effects of adiponectin, it will be of great interest to establish whether an overexpression of adiponectin can rescue parts of the phenotype we see, such as the decrease in insulin sensitivity and the steatotic liver, primarily observed in chronic HFD fed Tadipo mice. We are also interested in establishing whether we can promote healthy adipose tissue expansion and observe opposite effects when we re-express or overexpress TLR4 in the adipocyte. Our groups have generated an inducible TLR4 overexpression mouse model and a constitutively TLR4 re-activation mouse model. We are currently crossing the inducible overexpression model into the whole-body TLR4 knockout model and characterizing the re-activation model to further validate our working hypothesis and to extend our current understanding of the role of adipose inflammation in obesity-induced insulin resistance. An interesting experiment will be the injection of LPS into mice that lack TLR4 systemically with the exception of mature adipocytes. What will be the effect of this cell-type specific inflammatory model on fat mass and insulin sensitivity? Will it affect the hepatocyte, and if so, what is the time course of this phenomenon? Similarly, we can express TLR4 exclusively at the level of the pre-adipocyte using newly developed PDGF-R-rtTA mice that the Gupta lab has generated to determine whether inflammation targeted to these precursor cells enable the system to differentiate an increased number of adipocytes.

In summary, I have taken advantage of state-of-the-art genetic approaches to shed light on adiponectin maturation and the related inflammatory response in adipocytes. In the

process, more tools have been generated and additional important questions have arisen that our groups will be well positioned to address in the near future.