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Fatty Acid Fate In Determining Oxidation And Inflammation In Adipose Tissue

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FATTY ACID FATE IN DETERMINING OXIDATION AND INFLAMMATION IN ADIPOSE TISSUE

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DEDICATION

"Nel mezzo del cammin di nostra vita
mi ritrovai per una selva oscura
che' la diritta via era smarrita."
– Dante Alighieri La Divina Commedia – Inferno

"...ma gia volgena il mio disio e'l velle
si come rota ch'igualmente e mossa,
l'Amor che muove il sole e l'altre stelle"
– Dante Alighieri La Divina Commedia – Paradiso

To my wife, Sabrina, I thank you for your love and support down this long and arduous road. Your love truly does move the sun and the stars.

"e quindi uscimmo a riveder le stelle"
– Dante Alighieri La Divina Commedia – Inferno

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PREFACE

This thesis is based upon the following published papers:

1. **Mottillo E.P.**, X.J. Shen, J.G. Granneman. β3-adrenergic receptor induction of adipocyte inflammation requires lipolytic activation of p38 and JNK. ***BBA - Molecular and Cell Biology of Lipids***. Sep;1801(9):1048-55

2. **Mottillo E.P.** and J.G. Granneman (2011). Intracellular fatty acids suppress β-adrenergic induction of PKA-targeted gene expression in white adipocytes. ***Am J Physiol Endocrinol Metab***., 301(1), E122-31.

3. **Mottillo E.P.,** Block A.E., Leff T. and J.G. Granneman (2012). Lipolytic products activate peroxisome proliferator-activated receptor (PPAR) α and δ in brown adipocytes to match fatty acid oxidation with supply. ***J. Biol. Chem***. 287: 25038-48.

4. Lee Y.H*****, **Mottillo E.P.*** and J.G. Granneman (2013). Adipose Tissue Plasticity from WAT to BAT and in between. ***BBA Molecular Basis of Disease*****.** Invited review, in submission.

*****Equal contribution

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Chapter 1. Introduction

1.1 Adipose tissue and obesity

The incidence of obesity has been increasing over the past 20 years in the United States, with current rates greater than 27%, and is slowly becoming a global problem (Caballero 2007). Obesity is a major risk factor for cardiovascular disease, dyslipidemia, certain types of cancer, and is strongly correlated with type 2 diabetes mellitus (MMWR 2006). Although excessive adiposity is the most prominent feature of obesity, recent research indicates that disease risk is most related ability of adipose tissue (AT) to act as an energy buffer and not total fat mass (Adilson Guilherme et al. 2008). For instance, Cowden syndrome, which is due to mutations in phosphatase and tensin homolog, causes obesity, yet patients are insulin sensitive (Pal et al. 2012). Conversely, patients with familial partial lipodystrophy develop a loss of subcutaneous fat, along with severe insulin resistance and diabetes (Hegele et al. 2007). Similarly in rodents, genetic models that limit adipose tissue expansion during overnutrition promote ectopic lipid accumulation and exacerbate diabetes (Wang et al. 2008). On the other hand, increasing the storage capacity of WAT reduces ectopic triglyceride (TG) accumulation and improves insulin sensitivity (Kim et al. 2007).

Central to excess adiposity causing diabetes are free fatty acids (FFAs). Excess FAs are toxic to cells, therefore the ability of white adipose tissue (WAT) to store and control their release is essential. During obesity, excess FFAs lead to inflammation in WAT and whole body insulin resistance. Conversely, treatment with the anti-diabetic thiazolidinedione (TZD) drugs, limits FFA release by promoting fat storage, and improves insulin action, despite promoting greater weight gain (Seufert et al. 2004). The excess weight gain is not ideal in terms of the stigma

associated with obesity and the consequent poor quality of life. An alternative strategy to limit the effects of toxic FA is to promote their combustion within adipose tissue, providing the same beneficial effects, but without increased adiposity. This can be done by activating adipocyte β3-adrenergic receptors (AR) which stimulate thermogenesis in brown adipose tissue (BAT), increase whole body energy expenditure, and have anti-obesity and anti-diabetic effects in rodents (Ghorbani, Claus, and Himms-Hagen 1997, Grujic et al. 1997).

The mechanisms by which β3-ARs improve whole body metabolism are not completely understood, however, a central component is the mobilization of FFAs. In BAT, mobilized FFAs activate uncoupling protein 1 (UCP1), the molecular effector of thermogenesis, and provide fuel for sustained heat production. Thus, FFAs are necessary and sufficient to drive thermogenesis. Although acute mobilization of FFAs would be expected to promote inflammation in WAT, chronic activation of β3-AR appears to expand the ability of fat to oxidize fatty acids in situ, thereby lowering systemic FFAs and improving insulin action, while at the same time reducing adiposity. Thus, focusing on FA fate within WAT and BAT will be critical in understanding how activation of the β3-AR produces reciprocal responses of inflammation and oxidation.

1.2 Function of adipose tissue

Adipose tissue has long been considered an inert storage depot for excess energy; however, AT is now known to be a dynamic regulator of whole body insulin sensitivity and metabolism (Attie and Scherer 2009). AT normally functions as a buffer during energy imbalance, storing nutrients as TG in times of excess, and mobilizing FFAs in times of need. Because FFAs and their metabolic products can be toxic, homeostatic mechanisms exist to

finely balance lipid storage and mobilization so that potentially toxic lipids do not accumulate in peripheral organs (Unger 2002). The ability of adipose tissues to buffer variations in energy supply and demand is achieved by mechanisms which allow fat cells to sequester excess energy and mobilize it in times of deficit. In addition, AT can regulate whole body metabolism by releasing hormones, termed adipokines, that control energy intake as well as promote energy utilization in peripheral tissues (Prins 2002). As such, the function of the adipose organ (Cinti 2001) is to prevent detrimental accumulation of surplus energy in non-adipose tissues. However, during chronic overnutrition the buffering capacity and hormonal control of adipose tissue is impaired, resulting in the spillover of lipids from fat tissue and the pathological accumulation of lipids within key insulin sensitive tissues. The formation of ectopic lipid suppresses insulin sensitivity in muscle and liver, and insulin production by the pancreas in a process termed lipotoxicity (Unger 2002, Unger and Scherer 2010). Thus, one might anticipate that promoting lipid storage in adipose tissue or increasing FA oxidation might have beneficial effects on whole body metabolism.

1.3 White and brown adipose tissue

Historically, AT tissue has been divided into WAT and BAT based upon anatomical location, morphology and function. WAT is the main site for storage of TG and contains unilocular fat cells that have sparse mitochondria. WAT can be subdivided into subcutaneous depots found beneath the skin, and visceral (also called abdominal fat) depots found surrounding the internal organs. Visceral fat drains directly into the hepatoportal systems and is associated with poor metabolic profile and displays characteristics of inflammation in obese

rodents and humans. On the other hand, subcutaneous fat is thought to have a protective function in metabolic disorders (Gesta, Tseng, and Kahn 2007). In contrast to WAT, BAT is a thermogenic organ with dense sympathetic innervation and a well developed vasculature that functions to maintain body temperature in response to cold exposure (Cannon and Nedergaard 2004). BAT is located in the anterior and dorsal regions which reflects its function as the main heater organ in rodents and larger mammals (Symonds et al. 2011). Morphologically, BAT contains multilocular fat cells with abundant mitochondria containing UCP1. In contrast to WAT, FFAs mobilized in BAT activate UCP1 (Fedorenko, Lishko, and Kirichok 2012), and supply fuel for high rates of oxidative metabolism. Thus, lipolysis serves different functions in WAT vs. BAT.

1.4 Lipolysis and fatty acid fate in adipocytes

TGs are stored and released from organelles termed lipid droplets. Lipid droplets are the site of lipolysis, which is a highly dynamic and regulated process. Adipocyte lipolysis is mediated by the stimulation of the β-AR which triggers adenylyl cyclase (AC) to elevate cAMP levels and activate protein kinase A (PKA). PKA phosphorylates the lipid droplet protein perilipin 1 (Plin1), which releases the co-lipase abhydrolase domain containing 5 (Abhd5) and thereby activates adipose triglyceride lipase (ATGL) (Granneman and Moore 2008). PKA also phosphorylates hormone sensitive lipase (HSL) a major diacyglycerol (DAG) lipase (Kraemer and Shen 2002). In this fashion, ATGL releases the initial FA from the sn-2 position of TG to produce DAG, allowing HSL to release a FA and monoacylglycerol, in which monoglyceride lipase

releases the last FA and glycerol (Eichmann et al. 2012). In addition to being an energy source, FFAs can also mediate other biological effects.

FFAs liberated from TG within adipocytes can have multiple fates (Figure 1). During lipolysis, FFAs are primarily released from white adipocytes into systemic circulation where they can be utilized as an energy source by tissues such as skeletal muscle and heart. In order to undergo intracellular metabolism, FAs need to be first activated in an ATP dependent manner. This is done by acyl-CoA synthetase, forming a fatty-acyl-CoA, thus trapping FFAs within cells (Thompson, Lobo, and Bernlohr 2010). Once activated, FFAs are catabolized in the mitochondria via β-oxidation, or targeted toward glycerolipid or phospholipid formation. Furthermore, FFAs are also thought to be ligands for the peroxisome proliferator activated receptors (PPARs), which are transcription factors that regulate various aspects of lipid metabolism (Evans, Barish, and Wang 2004). In addition, FFAs generated by lipolysis impact cellular signaling by negatively regulating cAMP generation in cells (Fain and Shepherd 1979). Finally, FFAs are known to cause inflammation in WAT during β3-AR activation (Granneman et al. 2005) and are strongly implicated in the etiology of adipose inflammation during obesity (Adilson Guilherme et al. 2008).

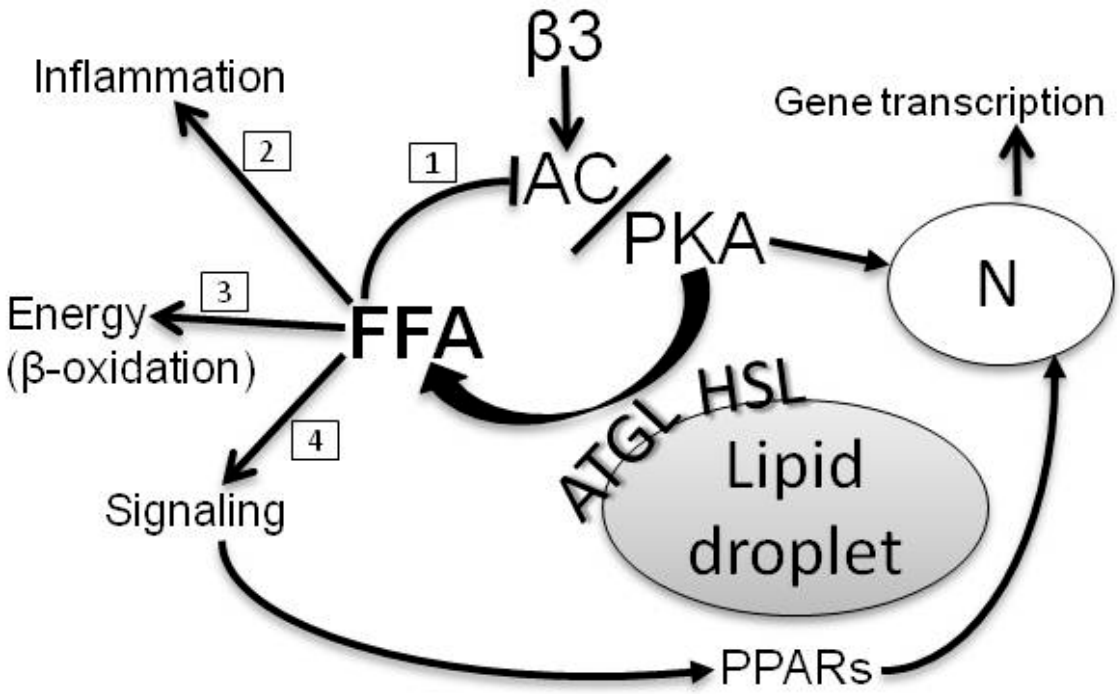

Figure 1. Fatty acid fates in adipocytes. Stimulation of β3-AR promotes PKA activity, leading to direct activation of gene transcription within the nucleus (N) and activation of lipolysis (ATGL and HSL). Fatty acids liberated by lipases have various fates within a fat cell: 1), FFAs can act in a negative feedback manner on adenylyl cyclase (AC); 2), can promote inflammation; 3), burned up as energy by via β-oxidation in mitochondria; or 4), act as signaling molecules for nuclear receptors.

1.5 Obesity and inflammation

The identification of obese adipose tissue as a source of pro-inflammatory cytokines prompted a plethora of research into how inflammation in AT precipitates diabetes (Weisberg et al. 2003). Numerous studies demonstrate that obesity in mice elevates the expression of inflammatory cytokines such as chemokine (C-C motif) ligand 2 (CCL2), plasminogen activator inhibitor-1 (PAI-1) and interleukin 6 (IL-6), and recruits of activated macrophages (Cinti et al. 2005, Lumeng, Bodzin, and Saltiel 2007, Weisberg et al. 2006, Weisberg et al. 2003).

Importantly, genetic and/or pharmacological interventions that reduce AT inflammation, improve systemic insulin sensitivity (Goldfine, Fonseca, and Shoelson 2011, Weisberg et al. 2006). While numerous mechanisms have been proposed as to how obesity causes inflammation in adipose tissue, the more accepted mechanisms include formation of hypertrophic fat cells and production of lipid mediators.

Chronic overnutrition increases the occurrence of hypertrophic fat cells, which upon reaching a critical size become distressed and undergo a necrotic/apoptotic cell death (Cinti et al. 2005). Distressed fat cells send out signals, such as CCL2, which recruits macrophages that form a syncytium surrounding fat cells, termed a crown like structures (CLS) (Lumeng, Bodzin, and Saltiel 2007, Weisberg et al. 2006). These macrophages are thought to be of the pro-inflammatory type and are an important source of inflammatory cytokines (Lumeng, Bodzin, and Saltiel 2007). The formation of CLS further triggers an inflammatory response in surrounding cells and impedes the buffering of FAs in fat cells of mice (A. Guilherme et al. 2008) and in cultured human cells (Wentworth et al. 2010). However, the contribution of fat cells to cytokine expression is not well understood. This systemic low-grade inflammation disrupts the balance between FA storage and mobilization in adipose tissue, which promotes ectopic lipid accumulation and subsequent production of lipid mediators in non-adipose tissue. For example, saturated FAs can activate the innate immune signaling pathway Toll-like receptor 4 (TLR4) to mount an inflammatory response, which can then suppress insulin action (Nguyen et al. 2007). However, the direct role of TLR4 in promoting insulin resistance remains controversial (Murumalla et al. 2012, Orr et al. 2012). In addition, metabolites of FAs such as

DAGs and ceramides can also promote inflammation and impair insulin signaling (Glass and Olefsky 2012, Holland et al. 2011).

A drawback of high fat diet studies is that they involve long term treatment over months to mediate their effects, and there is a strong dissociation between early change in fat cell function and the final outcome of adipose tissue inflammation and insulin resistance. However, the β3-AR provides a tractable model to understand how FFAs produce inflammation in WAT which occurs over hours. Thus, understanding the mechanisms of how activation of β3-AR produces inflammation and means of increasing lipid oxidation that limit toxic fatty acids could be useful in treating obesity and improving insulin action.

1.6 Therapeutic remodelling of adipose tissue by targeting β3-Adrenergic Receptors

As mentioned in the beginning of the chapter (1.1), one means of addressing the adverse metabolic consequences of obesity is to promote the combustion of FFAs within AT by activating β3-ARs. β3-AR agonists improve metabolism in rodent models of type 2 diabetes by expanding the ability of brown and white adipose tissues to oxidize FAs in situ, and thereby prevent systemic lipotoxicity. Although β3-AR agonists have not been successful in humans (Arch 2011, Arch 2008), this does not preclude their study in rodents. Their experimental use in rodents can uncover parallel mechanisms that can be targeted pharmacologically, which could have use in humans. Moreover, the recent identification that adult humans have significant BAT depots raises the possibility of utilizing BAT therapeutically (Ravussin and Kozak 2009, Ravussin and Galgani 2011).

In rodents, physiological or pharmacological stimulation of β3-ARs activates brown fat by mobilizing FFAs, thus sharply elevating FA oxidation and metabolic rate (Arch et al. 1984, Yoshida et al. 1994). The anti-diabetes effects of β3-AR agonists require chronic treatment (Yoshida et al. 1994), and are correlated with dramatic remodeling of WAT (Granneman et al. 2005, Li et al. 2005). Initially, activation of the β3-AR produces a transient inflammatory state in WAT, marked by the expression of inflammatory cytokines and recruitment of myeloid cells (Granneman et al. 2005). However, with chronic stimulation the inflammation wanes as WAT is transformed from a lipid storage organ, into one that can oxidize FAs. This "browning" of WAT is marked by the appearance of numerous multilocular brown adipocytes (BAs), and the upregulation of genes involved in FA oxidation and mitochondrial biogenesis (Granneman et al. 2005). Understanding the mechanisms of β3-AR is of importance since activation of BAT, and browning of WAT (Vegiopoulos et al. 2010) can have anti-obesity effects and improve glucose homeostasis and insulin sensitivity in rodents (Ghorbani, Claus, and Himms-Hagen 1997, Grujic et al. 1997). Moreover, the recent identification that human BAs are more similar to BA recruited in rodent WAT (Sharp et al. 2012, Wu et al. 2012), suggests that browning of WAT may be of therapeutic benefit.

The mechanisms by which β3-ARs enhance the catabolic character of brown fat and the browning of WAT are not completely understood, but likely involve synergy among various signalling pathways (Collins, Yehuda-Shnaidman, and Wang 2010, Kozak 2011). Many of the factors involved in enhancing BAT thermogenesis, or promoting the browning of WAT, likely lie downstream of the cAMP/PKA signalling node (Cao et al. 2004, Kozak 2011, Rim and Kozak 2002). One such critical factor is peroxisome proliferator-activated receptor gamma coactivator

1α (PGC1α), which is a transcriptional co-activator of nuclear receptors (Puigserver et al. 1998). PGC1α was initially described as a cold-induced mRNA transcript, and is necessary for the maximal induction of UCP1 mRNA, and is a master regulator of mitochondrial biogenesis (Chang et al. 2012, Puigserver et al. 1998, M. Uldry et al. 2006). Importantly, PGC1α in adipose tissue is critical for cold-adaptive thermogenesis (Chang et al. 2012, Kleiner et al. 2012). In addition, PGC1α and peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor that is involved in lipid signaling, have been identified as genes associated with maximal induction of UCP1 expression in WAT depots (Xue et al. 2005).

Stimulation of the β3-AR/PKA pathway promotes the transcription of thermogenic genes such as PGC1α and UCP1 genes. Exactly how β-ARs promote thermogenic gene transcription is not entirely known, but PKA phosphorylates CREB (Reusch, Colton, and Klemm 2000, Rim and Kozak 2002), thereby activating transcription via cAMP response elements (CRE) (Karamitri et al. 2009). In addition, the genes for UCP1 and PGC1α contain binding sites for the PPAR nuclear receptors.

1.7 Peroxisome proliferator-activated receptors (PPARs)

The nuclear receptors PPARα, PPARδ and PPARγ are activated by various lipid species and regulate FA oxidation and lipid storage (Lee, Olson, and Evans 2003). PPARγ is highly expressed in adipose tissue (Chawla, Schwarz et al. 1994) and is a master regulator of adipocyte differentiation (Tontonoz, Hu et al. 1994; Hu, Tontonoz et al. 1995; Lehmann, Moore et al. 1995) (reviewed in Spiegelman 1998). Thus, expression and activation of PPARγ is necessary and sufficient to induce adipogenesis in cultured embryonic fibroblasts (Rosen, Sarraf et al.

1999). While the endogenous ligand(s) of PPARγ are not certain (Kliewer, Sundseth et al. 1997), it is clear that TZDs are potent and selective agonists of this receptor (Lehmann, Moore et al. 1995; Berger, Bailey et al. 1996) (reviewed in Spiegelman 1998). In humans, TZDs ameliorate insulin resistance and increase peripheral glucose utilization and uptake in various target tissues and improve serum lipid levels (Seufert, Lubben et al. 2004). On the other hand, PPARα is highly expressed is oxidative tissues such as heart, liver and BAT. Consistent with this pattern of expression, PPARα has a critical role in controlling FA oxidation and is the target for the lipid-lowering agents fibrates (Evans, Barish, and Wang 2004). Much less is known about PPARδ which is ubiquitously expressed in humans and rodents (Ahmed et al. 2007, Evans, Barish, and Wang 2004); however there is a growing appreciation that PPARδ also regulates FA oxidation. Overexpression of PPARδ upregulates the expression of genes involved in thermogenesis and protects mice from diet-induced obesity (Wang et al. 2003). Thus it seems that PPARs have opposing function on lipid metabolism with PPARγ promoting the storage of lipids, while PPARα and δ promoting lipid oxidation. Interestingly, long-chain FAs are potent activators of PPARα and δ in vitro, but have weak to no activity on PPARγ (Forman, Chen, and Evans 1997). While the therapeutic benefits of targeting the PPARs are widely known (Ahmed et al. 2007), how these receptors are activated in a biological context and the cellular site(s) of ligand production are less well understood. However, growing evidence suggests that lipolytic products are important source of PPAR ligands.

1.8 Lipolysis and PPAR signaling

The earliest indication that activating lipolysis could alter PPAR activity was from studies of the β3-AR agonists. Activation of the β3-AR stimulates lipolysis and also promotes the expression of PPAR target genes (Granneman et al. 2005), however, whether the pathways of lipolysis and gene transcription interact, is not known. Subsequent genetic manipulations that increase lipolysis such as loss or overexpression of Plin1 (Castro-Chavez et al. 2003, Sawada et al. 2010) or overexpression of ATGL (Ahmadian et al. 2009), enhances the catabolic phenotype of WAT with increased mitochondrial mass and upregulation of genes involved in FA oxidation. In contrast, Mice lacking ATGL in AT have reduced expression of PPARα target genes in BAT (Ahmadian et al. 2011). Furthermore, the upregulation of genes involved in lipid catabolism by β3-AR activation is delayed in WAT of mice lacking HSL (Mottillo, Shen, and Granneman 2007). In other tissues ATGL is needed to maintain an oxidative phenotype in the heart (Haemmerle et al. 2011), and Abhd5 regulates skeletal muscle lipolysis and oxidative metabolism in a PPARδ-dependent manner (Badin et al. 2012). Overall, these studies suggest that increasing lipolysis may be beneficial, provided that oxidative tissues, including adipose tissue, are able to counter excess FFA flux by increasing FA oxidation and/or handling. However, a direct demonstration that lipolysis can alter PPAR activity is lacking. Furthermore, the above studies did not address whether lipases can produce direct ligands that modify the transcriptional activity of PPARs.

FAs are toxic and the inability to counter excess FFAs can produce inflammation. This relationship is best exemplified in PPARα KO mice that have been challenged with the β3-AR agonist. Normally activation of the β3-AR produces a transient inflammatory response in WAT that wanes as FA oxidation is increased in situ. PPARα KO mice display a constant and

heightened inflammatory state in white fat in response to continuous β3-AR agonist treatment and are unable to upregulate a FA oxidation program (Li et al. 2005). The upregulation of lipid oxidation in WAT is chronic adaption that balances flux with oxidation, however, counter responses might exist to acutely limit FFAs and prevent excessive mobilization. One such mechanism could be the ability of FFAs to inhibit the production of cAMP. On the other hand, FFAs drive thermogenesis in BAT in which lipolysis is balanced with oxidation. However, very little work has been done to understand how FA fate differs between WAT and BAT. Thus, by focusing on the fate of FAs, the work proposed below will provide an integrative comprehension of how the β3-AR produces contrasting phenomena in WAT and BAT.

1.9 Hypothesis and aim of the project

The organizing hypothesis of this work is that the differential response produced by β3-AR activation in WAT and BAT is dependent on the fate of FFAs. Our previous work that activation of the β3-AR produces inflammation in WAT, and current findings lead to the following specific hypothesis: 1) intracellular FAs mobilized by lipolysis promote the expression of inflammatory cytokines in white adipocytes. 2) Intracellular FAs are feedback inhibitors of the cAMP/PKA pathway to limit the expression of PKA target genes. 3) Lipolysis in brown adipocytes (BAs) and BAT activates the nuclear receptors PPARs to promote lipid oxidation.

The goal of this dissertation is to understand the signalling mechanisms of lipolysis, and to identify how FFAs mediate the differential responses in WAT and BAT. This hypothesis will be tested by three specific aims: 1) to understand the mechanisms by which FAs promote the expression of inflammatory cytokines. 2) To determine the role of lipolysis in the expression of

oxidative genes in WAT and the browning of WAT. 3) To determine the role of lipolysis in promoting an oxidative phenotype in BAT.

Overall, the above aims are part of a main objective to understand how metabolic pathways interact with transcriptional events in adipose tissue, and the functional significance of these interactions. In a broader sense, these objectives will help determine if activating lipolysis might be of therapeutic benefit, and if targeting lipolysis pharmacologically is a feasible approach in a clinical setting. Data generated from these experiments will improve our understanding of adipocyte biology, providing valuable information on the differential roles of lipolysis in mediating inflammation and oxidation. Finally, data obtained from these studies may contribute to the development of new anti-obesity therapies that which promote an oxidative phenotype in WAT and activation of BAT in humans.

Chapter 2. Materials and Methods

2.1 Animal studies

All animal protocols were approved by Institutional Animal Care and Use Committee (IACUC) and the Division of Laboratory Animal Resources (DLAR) at Wayne State University. HSL-KO mice were supplied by Dr. F. Kraemer (Stanford University). $HSL^{+/-}$ mice on a C57Bl/6 (BL6) background were bred at Wayne State University. Mice were genotyped by PCR using primers HSL-A, 5'AGAGAGACCAACTCAGCCTCCCAC-3'; HSL-B, 5'CAAGGTGTCTGTCTGTGCTGTCTT-3'; and SI-166, 5'AGGATTGGGAAGACAATAGCAGGCAT-3'; where PCR of the WT allele generates a 269-base pair (bp) band and the mutant allele a 320-bp band. Male or female mice were injected intraperitoneally (IP) with the β3-AR agonist (CL 316,243; CL, 10 nmol), and 6 h later, epididymal white adipose tissue (EWAT) and BAT were collected and stored in RNALater (Ambion) at -80 °C.

To examine the effects of pharmacological inhibition of HSL, 8 week old male BL6 mice (n=7-8) were pretreated with 30 mg/kg of the selective HSL inhibitor BAY 59-9435, 4-isopropyl-3-methyl-2-[1-(3-(S)-methyl-piperidin-1-yl)-methanoyl]-2H-isoxalo-5-one (BAY) (Claus et al. 2005), suspended in 0.5% methylcellulose or methylcellulose alone via oral gavage. After one hour, mice were injected IP with 10 nmol of CL or H_2O, and sacrificed 3 hr later and EWAT or BAT was removed and stored as above. For measurement of plasma FAs and glycerol, blood was collected via retro-orbital bleed 45 min after CL treatment. Studies to examine the sub-acute effects of HSL inhibition were performed on male BL6 mice and treated daily for five days with BAY (30mg/kg) or MC via gavage, followed by an injection of CL (10 nmol) or saline 1 h later. Body composition was determined by NMR (EchoMRI) prior to drug treatment (day 0)

and after 5 days. In situ electron transport chain activity was examined in EWAT minces by measuring the reduction of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma), as previously described (Li et al. 2005).

2.2 Cell culture and in vitro assays

The 3T3-L1 cell line was used as a cell culture model for adipocytes. The cells were originally derived from Swiss mouse embryos by Dr. Howard Green (Green and Kehinde 1975). Cells as fibroblasts were obtained from ATCC (CL-173) and maintained in DMEM with 10% bovine calf serum (BCS). Fibroblasts were differentiated as previously described (Moore et al. 2005). Briefly, cells were grown to confluency in BCS. Two day post-confluency (Day 0), media was changed to Differentiation I media (DMEM 10% fetal bovine serum [FBS], 1 ug/ml insulin, 0.5 mM IBMX [3-isobutyl-1-methylxanthine], 1 uM dexamethasome) and maintained for three days. Media was then changed to Differentiation media II (DMEM 10% FBS 1 ug/ml insulin). Two days after, cells were maintained in DMEM 10% FBS, and experiments were performed on adipocytes with greater than 90% differentiation on days 13-14. One day prior to the experiment, adipocytes were cultured overnight in DMEM (Invitrogen), and media was replaced with serum-free, phenol red-free, DMEM (Mediatech) containing 0.1 % FA free bovine serum albumin (BSA) (Roche Diagnostics) unless indicated otherwise. Differentiated adipocytes were pretreated for 1 h with the selective HSL inhibitor BAY (5 µM), inhibitor of serine palmitoyltransferase (SPT) (Myriocin, 10 µM), or vehicle (DMSO) then stimulated with 10 µM isoproterenol or H_2O (control) for 3 h. In other experiments cells were treated with the HSL inhibitor BAY (5 µM) or DMSO for 30 min followed by forskolin (FSK) (4 µM; Sigma) or the cAMP

analogue 8-Br-cAMP (1 mM; Sigma) for 3 h. Long-chain acyl-CoA synthetase (ACSL) activity was inhibited with triacsin C (5 µM; Sigma) for 30 min followed by isoproterenol treatment (10 µM, 3 h).

A BA cell line derived from mouse fetal brown fat and subsequently transformed with SV40 large T antigen, was cultured and differentiated as previously described (Marc Uldry et al. 2006). Briefly, confluent cells were placed in induction media (0.5 mM IBMX); 0.25 mM indomethacin; 2 µg/ml dexamethasone; 1 nM T3, 20 nM insulin) for two days and subsequently maintained on differentiation media (1 nM T3, 20 nM insulin). All experiments were performed on cultures 6-8 days post-induction. Unless otherwise indicated, cells were rinsed with PBS and media was changed to HEPES-buffered Krebs Ringer buffer (HKRB) + 1% BSA. Where indicated, BAs were pretreated with triacsin C (5 µM; Sigma), BAY (5 µM), etomoxir (50 µM; Sigma), GW6471 (10 µM; Tocris), GSK0660 (2 µM; Tocris) or GW9662 (30 µM; Tocris), followed by isoproterenol (10 µM, Sigma) or 8-Br-cAMP (1 mM, Biolog). For experiments examining mitochondrial gene expression, BAs were stimulated for 24 h with 8-Br-cAMP (1 mM) or selective agonists of PPARα (GW7647, 1 µM; Tocris), or PPARδ (L-165,041, 5 µM; Tocris).

2.3 Liquid chromatography–mass spectrometry (LC-MS)/MS quantification of ceramides and sphingolipids

3T3-L1 adipocytes were stimulated with isoproterenol for 3 h, and cell pellets and culture media were collected and processed for lipids quantification by LC/MS technique. Reverse phase HPLC was performed using BDS HYPERSIL C8 columns (Thermo Scientific) and gradient

elution on Waters Alliance 2695 system (Waters Corporation). The mobile phase consisted of methanol, water, and ammonium formate. Solvent A was 2 mM ammonium formate in methanol with 0.2% formic acid. The column was equilibrated with solvent A for 5 min. Samples were injected using a Waters autosampler maintained at 10 ± 2^{o}C. The injection volumes were 80 µl for each sample. A complete injection of each sample took 7 min including column equilibration, while the flow rate was 0.3 ml/min. The HPLC eluent was directly injected into a QuattroLC mass spectrometer (Micromass-Waters, MA), equipped with an electrospray ion (ESI) source that was used for ESI–MS/MS. The ESI–MS/MS experiments for the quantification of sphingolipids were carried out in the positive ion mode with ESI needle voltage, 2.8 kV; source block temperature, 120^{o}C; desolvation temperature, 350^{o}C; desolvation gas flow, 540 l/h; nebulizer gas flow, 80 l/h; and the collision gas pressure was 3.2×10^{-4} bar. Cone voltage and collision energy for each multiple reaction monitoring (MRM) transition were optimized. Chromatographic data were analyzed by Quanlynx module of the Masslynx software (Waters Corporation, MA) to integrate the chromatograms for each MRM transition.

2.4 cAMP measurements

For measurement of cAMP, 3T3-L1 adipocytes were pretreated with BAY or triacsin C and stimulated for 10 min with isoproterenol. Cells were lysed with 0.1 M HCl, 0.2% Triton-X-100. Acidified cell lysates were collected and neutralized with NaOH and centrifuged at 1000×g for 10 min and the supernatant was collected. The cAMP levels were quantified by ELISA (Biomedical Technologies) as suggested by the manufacturer.

2.5 siRNA knockdowns

Knockdown of ATGL in 3T3-L1 adipocytes was performed using small interfering RNA (siRNA), as previously described (Kilroy, Burk, and Floyd 2009). Briefly, adipocytes were trypsinized and replated (1.8×10^5 cells/well) in collagen-coated 24-well plates containing 100 nM siRNA against ATGL (siATGL; Dharmacon M-040220-01) or non-targeting siRNA (siCON; Dharmacon D-001210-01). At 72 h post-transfection, media was changed and cells were stimulated with 10 µM isoproterenol for 3 h in triplicate per group.

siRNA knockdown in BAs was performed on cultures four days post-induction. Cells were trypsinized and replated (2.4×10^5 cells/well) in collagen-coated 24-well plates containing Lipofectamine 2000 (Invitrogen) and 50 nM of SMARTpool siRNA (Dharmacon) against PPARα (siPPARα; M-040740-01-0005), PPARδ (siPPARδ; M-042751-01-0005) or non-targeting siRNA (siCON; D-001210-01). Experiments were performed 72 h post-transfection on triplicate wells.

2.6 Lentivirus transduction

Undifferentiated BAs were infected with lentivirus vectors (Open Biosystems) for a control non-targeting shRNA (RHS4346; shCON) or one directed against ATGL (RMM4431-98739845; shATGL) at a multiplicity of infection of 100 for 24 h, and selected by hygromycin (GoldBio, 4 µg/ml) for 1 week followed by GFP fluorescence by FACS. Cells were differentiated and treated as above.

2.7 Reporter assays

cAMP response element (CRE) reporter assays were performed using a cAMP responsive reporter plasmid (pADneo2 C6-BGL) provided by Dr. Adolf Himmler (Himmler, Stratowa, and Czernilofsky 1993). Five days after induction of differentiation, 3T3-L1 adipocytes were trypsinized and replated (3.6×10^5 cells/well) in collagen-coated 12-well plates containing media and 2 µg of pADneo2 C6-BGL, 0.08 µg of β-galactosidase (β-gal) and 4 µl Lipofectamine 2000 (Invitrogen). On the following day, transfection media was changed to DMEM containing 0.1% BSA, and cells were treated in duplicate with BAY (5 µM), triacsin C (5 µM) or DMSO for 30 min, followed by treatment with FSK (10 µM) for 6 h. Cells were harvested in 150 µl of Cell Culture Lysis Reagent (Promega) and 20 µl was assayed in duplicate. Luciferase activity was measured in Luciferase Assay Buffer (15mM KH_2PO_4, 15mM $MgSO_4$, 4mM EDTA, 2mM ATP, 1mM dithiothreitol) with 15 µg of D-Luciferin (Gold Biotechnology) using a MicroLumatPlus LB96V luminometer (Berthold Technologies). β-gal activity was measured in 100 mM sodium phosphate buffer pH 7.3, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol with ONPG (*o*-nitrophenyl-β-D-galactopyranoside) at 420 nm (Versamax; Molecular Devices). Luciferase activity was normalized to β-gal and 4 separate experiments were analyzed by two-way ANOVA. Results are reported as fold of FSK.

Gal4 luciferase reporter assays were performed by transfecting BAs with 0.7 µg of Gal4-PPARα or Gal4-PPARδ, 0.7 µg of luciferase reporter (pUAS-Luc2; Addgene # 24343) and 100 ng of β-gal reporter, as stated above. Cells were cultured on 12 well collagen-coated plates and used 6-7 days post-differentiation. Transfected cells were treated with BAY or DMSO for 30 min followed by Iso for 8 h, and lysed in 120 µl of Cell Culture Lysis Reagent (Promega). Ligands for

PPARα (Wy) and δ (L165) were used as positive controls. Luciferase assays were performed as described above and expressed as a % Iso.

2.8 Generation of reporter constructs

The ligand binding domain of human PPARα (NP_001001928; amino acid [a.a] 191-467), mouse PPARα (NP_035274; a.a 191-469) PPARδ (NP_035275; a.a. 162-441) and PPARγ (NP_001120802; a.a 207-474) were amplified by PCR. Ligand binding domains were cloned into AgeI/NotI on the C-terminus of full length Plin1 (NP_783571). The LxxLL containing domain of steroid receptor co-activator 1 (SRC1) (NP_035011; a.a. 620-770) was amplified from mouse cDNA and cloned in-frame into enhanced yellow fluorescent protein (EYFP)-C1 (Clontech) vector (EYFP-SRC1). Chimeras for the yeast GAL4 DNA-binding domain (DBD) were made by fusing the DBD of GAL4 (a.a 1-147) in frame with the ligand binding domain of human PPARα (a.a 167-468) or human PPARδ (NP_006229; a.a 139-441) into pcDNA3 (Invitrogen).

2.9 Live cell imaging

Fluorescent reporter experiments were performed by transfecting BAs with reporter plasmids for PPARα, or δ, or γ and EYFP-SRC1 with Lipofectamine LTX/Plus (Invitrogen), as recommended by the manufacturer. Cultures at 4-5 days post-induction were trypsinized, and seeded onto 25-mm coverslips containing a mixture of DNA, LTX/Plus and DMEM + 10% FBS. Images were acquired as previously described for EYFP fluorescence using a 40x 0.9NA water immersion lens (Granneman et al. 2009). Images for EYFP and phase contrast were acquired every minute and the region of interest (ROI) from an average of two frames was quantified

using IPlabs software (Scanalytics). Cells were pretreated with DMSO or BAY for 10 min and the basal fluorescence was recorded for 4-6 frames, followed by stimulation with 8-Br-cAMP (1mM) for 20 min, and finally by the addition of ligands for PPARα (Wy 14,643 (Wy); 100 µM), PPARδ (L-165,041 (L165); 10 µM) or PPARγ (Rosiglitazone [Rosi]; 10 µM) for 8-12 min. The data from the ROI was normalized to the maximum EYFP fluorescence in the ROI induced by Wy, L165 or Rosi. The effect of oleic acid (OA) on fluorescent reporters was tested with 400 µM OA, complexed to BSA, for 12 min. Data was normalized to the maximal effect induced by full PPAR agonists (Intrinsic Activity, IA), determined at the end of each experiment. Normalized data from 3-4 independent experiments with 2-4 coverslips per experiment were combined for presentation and statistical analysis. At the end of some experiments, coverslips were stained for neutral lipids with LipidTOX Deep Red (Invitrogen) and imaged with rhodamine excitation/emission filters (Granneman et al. 2009).

2.10 Quantification of cellular FFAs

Cellular FFA levels were quantified by briefly rinsing cells in 12 well plates once with DMEM containing 0.1% BSA, followed by two washes in PBS. Cellular FFAs were then extracted by adding 500 µl of Dole reagent (isopropanol/n-heptane/1N sulphuric acid: 40:10:1) and incubating for 10 min. Extractions were then transferred to 12x75 mm glass tubes and 200 µl of n-heptane followed by 300 µl of water was added. Samples were briefly vortexed and centrifuged at 1000 g for 5 min. 200 µl (62.5%) of the upper organic layer was then transferred to a new glass tube and samples were dried with a gentle stream of nitrogen gas while heating

at 40 C. Samples were then vortexed and resuspended in 110 µl of DMEM with 1% BSA and quantified as described below for FFAs.

2.11 Quantification of FFAs and glycerol by biochemical methods

FFA released into medium were quantified using a NEFA-HR(2) kit (Wako Chemicals, USA) as suggested by the manufacturer. Glycerol levels were quantified using Glycerol Reagent (Sigma-Aldrich) and both assays were read on a Versamax microplate reader (Molecular Devices).

2.12 RNA extraction and gene expression analysis

For analysis of mRNA, RNA from adipose was extracted in Trizol (Invitrogen) and then purified with an RNeasy mini kit (Qiagen). RNA from 3T3-L1 adipocytes and BAs was isolated using a Nucleospin RNA II kit (Macherey-Nagel). The expression pattern of various genes was quantified by qRT-PCR analysis, as previously described (Mottillo, Shen, and Granneman 2007). Briefly, RNA (0.5–1.0 µg) was reverse transcribed into cDNA by using Superscript II or Superscript III (Invitrogen) and oligo(dT) primers as recommended by the manufacturer. Thirty to fifty ng of cDNA was analyzed in a 20 µl quantitative PCR reaction (ABsolute Blue QPCR SYBR; ThermoScientific) with 80 nM of primers. Expression data were normalized to the house-keeping gene peptidyl-prolyl cis-trans isomerase A (PPIA) using the delta-delta CT method ($2^{-\Delta\Delta CT}$)(Livak and Schmittgen 2001). PPARα cDNA was amplified using forward and reverse primers 5′-CTAACCTTGGGCCACACCT-3 and 5′-CGGGTAACCTCGAAGTCTGA-3′, respectively. IL-6 cDNA was amplified using forward and reverse primers 5′-AGTGGCTAAGGACCAAGACC-3; and 5′-TCTGACCACAGTGAGGAATG-′3, PAI-1 cDNA were quantified with 5′-CCTCTTCATGGGCCAAGT-

3’ and 5’-GGTAAGGAGGAGTTGCCTTC -3. PDK4 cDNA was amplified using primers 5’-AGGATTACTGACCGCCTCTT-3’ (forward) and 5’-CGTCTGTCCCATAACCTGAC-3’ (reverse), MCAD with 5’-ATTGCCAATCAGCTAGCCAC-3’ (forward) and 5’-CTGATAGATCTTGGCGTCCC-3’ (reverse), COXII with 5’-CGAGTCGTTCTGCCAATAGA-3’ (forward) and 5’-TCAGAGCATTGGCCATAGAA -3’ (reverse), Cycs with 5’-GGAGAAAGGGCAGACCTAAT-3’ (forward) and 5’-CTGTCCAACAAAAACATTGCT-3’ (reverse)and COXIV with 5’-CCCTCATACTTTCGATCGTG -3’ (forward) and 5’- TTATTAGCATGGACCATTGGA -3’ (reverse). The mRNA knockdown of PPARα was detected with primer 5’-AGGCTGTAAGGGCTTCTTTC-3’ (forward) and 5’-CGAATTGCATTGTGTGACAT-3’ (reverse), and knockdown of PPARδ with 5’-GACAATCCGCATGAAGCTC-3’ (forward) and 5’-GGATAGCGTTGTGCGACAT-3’(reverse). All other cDNAs were amplified using primers previously described (Granneman et al. 2005, Li et al. 2005, Mottillo, Shen, and Granneman 2007, Mottillo and Granneman 2011).

2.13 Protein isolation and western blot analysis

Proteins were extracted in RIPA lysis buffer (25mM Tris pH 7.5, 150 mM NaCl, 1% Triton-X-100, 0.5%, Na-deoxycholate, 1% NP-40, 0.5% sodium dodecyl sulfate, and 1mM EDTA) containing protease inhibitors (Roche) and phosphatase (Pierce) inhibitors. Lysates were solubilized for 15 min at 4°C and centrifuged at 16,000 × g for 10 min to clear lysate. The extracts were recovered and proteins were quantified by using the bicinchoninic acid method (Pierce). Adipose tissue was homogenized in RIPA lysis buffer (20mM Tris, 150 mM NaCL, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 1mM EDTA) with inhibitors and treated as above. 50 µg of total protein was loaded on 10% mini-gels (Pierce) and SDS-PAGE was performed

under reducing conditions. Resolved proteins were transferred to PVDF and membranes were immunoblocked for 1 hr at room temperature in 5% powdered skim milk. Western blot was performed using antibodies against ATGL (Granneman et al. 2011), HSL (provided by Dr. A. Chaudhry) , and GAPDH (Millipore) as described (Mottillo, Shen, and Granneman 2007). Blots were then washed, incubated with a secondary donkey anti-rabbit HRP (Jackson immunological) diluted 1:5000, and visualized with SuperSignal West Dura substrate (Pierce). Digital images were captured to ensure that pixels were not over-saturated using a BioRad Quantity One imaging system.

2.14 Quantification of mitochondrial DNA content

Mitochondrial DNA was quantified on brown adipocytes that were stimulated with 8-Br-cAMP, as stated above. DNA was extracted with phenol-chloroform-isoamylalcohol and QPCR was performed using ABsolute Blue QPCR SYBR (ThermoScientific) for primers of Ndfuv1 (nuclear DNA, nDNA) and COXI (mitochondrial DNA, mtDNA), as described (Guo et al. 2009).

2.15 Measurement of mitochondrial respiration

Mitochondrial respiration was measured in digitonin permeabilized cells using the Mitoxpress (Luxcell) phosphorescent oxygen sensitive fluorescent probe (Jonckheere et al. 2010, Kuznetsov et al. 2008, Will et al. 2007). Probe fluorescence is quenched in the presence of molecular oxygen via a non-chemical (collisional) mechanism and is fully reversible. As cells respire the change in oxygen consumption is seen as an increase in probe fluorescence and reflects increase in mitochondrial activity over time (Will et al. 2007). shCON or shATGL BAs

were stimulated with 1 mM 8-Br-cAMP for 48 h with media changed every 24 h. Cells were trypsinized, washed once with PBS + 1 mM EDTA, followed by PBS + 1 mM EDTA + 1% BSA, and resuspended in 250 µl of Respiration Buffer B (Modified from (Kuznetsov et al. 2008); 0.5 mM EGTA, 5 mM $MgCl_2$, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM Hepes, 0.1 % BSA, 60 mM KCl, 110 mM Mannitol, pH 7.1). Cells were added to duplicate wells in a black clear bottom 96-well plate containing 110 µl of Respiration Buffer B plus 20 µg/ml digitonin, 100 nM Mitoxpress probe, 5 mM malate, and the indicated substrates (5 mM pyruvate, or 50 µM palmitoyl-carnitine, with 2 mM ADP or 10 µM carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP). The plate was covered with 100 µl of mineral oil to prevent diffusion of ambient oxygen and wells read with a Molecular Devices SpectraMax M5 plate reader in time-resolved mode (30 °C every 1.5 min, with an excitation/emission spectra of 380/650 nm, delay of 50 µs and a gate time of 200 µs). No oxygen consumption was observed in cells without substrate or in cells treated with substrate and 1 µM rotenone. Relative Oxygen Consumption Rate (ROCR) was calculated as the maximal linear increase in fluorescence over 4.5 min (SoftMax Pro, Molecular Devices) and normalized to 4×10^5 cells.

2.16 Statistical analysis

Unless stated otherwise, results are expressed as means ±SEM from a minimum of three independent experiments. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software) using one-way or two-way ANOVA. Planned post-hoc comparisons for one-way or two-way ANOVA were performed using Bonferroni post t-test (equal variances) or Dunn's multiple comparison test (unequal variances), as indicated in the legends.

Chapter 3. Intracellular fatty acids promote the expression of inflammatory cytokines in white adipocytes

3.1 Expression of inflammatory cytokines by β-AR activation is mediated by intracellular fatty acids

Our earlier work demonstrated that WAT inflammation induced by β3-AR agonist treatment is mediated by HSL activity. HSL is an adipocyte lipase that acts on DAG as its substrate, and is responsible for liberating up to 60% of FFAs in response to PKA activation (Kraemer and Shen 2002). HSL is required for the initial expression of inflammatory cytokines and subsequent recruitment of myeloid cells (Mottillo, Shen, and Granneman 2007). We suspected that HSL mediates inflammation by releasing FAs; however inflammation produced by β3-AR activation does not involve the putative extracellular saturated FA receptor, TLR4. To explore alternate mechanisms, we asked whether the expression of inflammatory cytokines might occur by intracellular accumulation and signalling of FFAs. To address this question, BSA, which is a serum factor that binds fatty acids, was systematically reduced in the media of cultured 3T3-L1 adipocytes in order to limit FFA efflux and promote intracellular accumulation (Vallano, Lee, and Sonenberg 1983). Reducing the concentration of BSA in the media systematically lowered extracellular FFA concentration while increasing intracellular FFA levels (Figure 2A and 2B). Lowering BSA greatly enhanced the expression of CCL2 and PAI-1. Importantly the expression of cytokines was the highest when cellular FFAs were the greatest (*i.e.*no BSA present) (Figure 2C). We also tested the effect of HSL on cytokine expression with BAY, a highly selective inhibitor that does not inhibit the other adipocyte TAG lipase, ATGL, and has no effect in HSL-null mice (Mottillo, Shen, and Granneman 2007). The increase in

intracellular FAs and the subsequent induction of CCL2 and PAI-1 was completely abolished by inhibition of HSL (Figure 2C), further supporting our initial finding that HSL regulates the expression of inflammatory cytokines in adipocytes (Mottillo, Shen, and Granneman 2007).

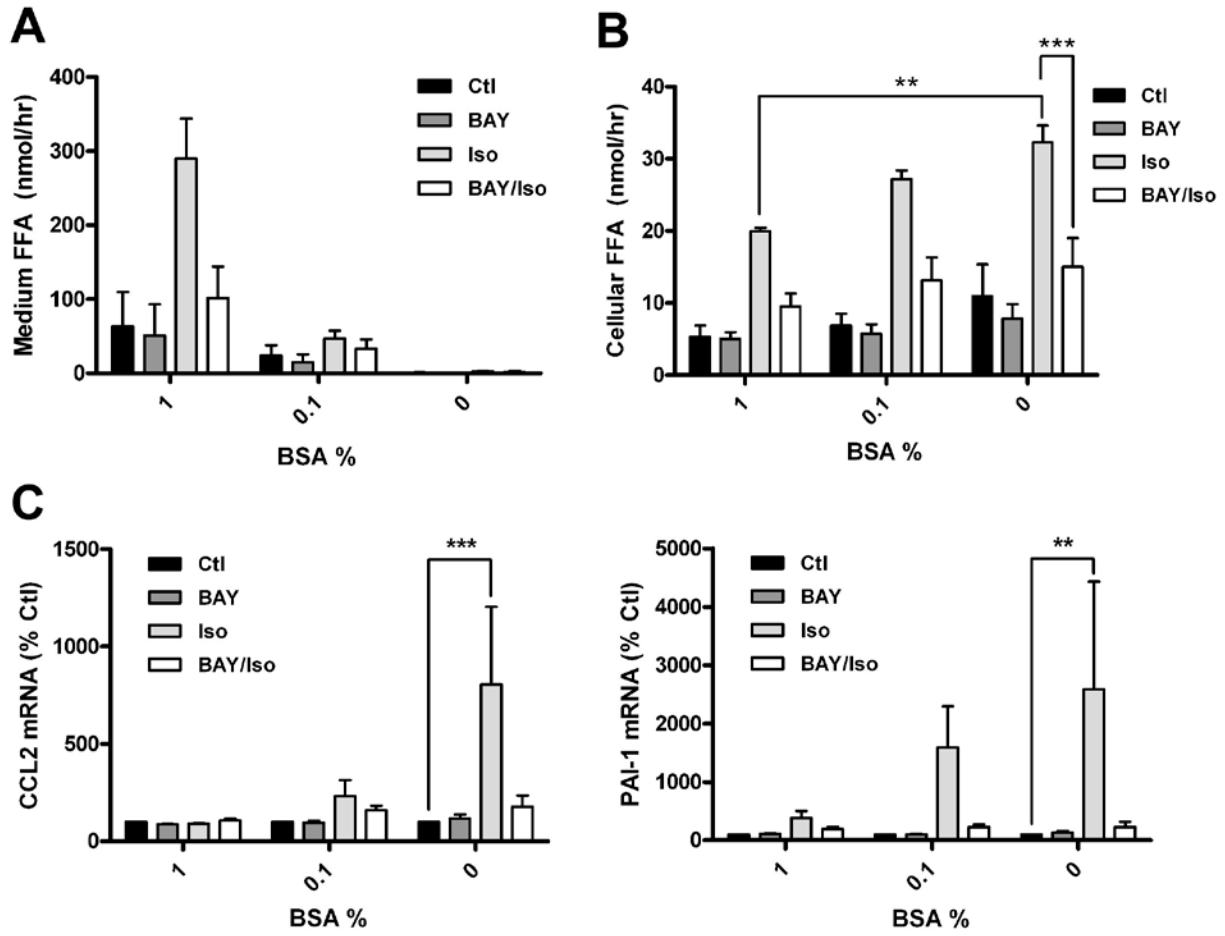

Figure 2. Intracellular FFAs promote the induction of CCL2 and PAI-1 by β-AR activation. A), 3T3-L1 adipocytes were incubated in decreasing concentration of BSA, pretreated with BAY and treated with Iso for one hour. B), cells from A) were washed once with 0.1% BSA, washed twice with PBS and fatty acids were extracted and quantified. Data shown are from three separate experiments. One-way ANOVA was performed with post t-test to indicate significance among indicated comparisons (** P<0.01, *** P<0.001). C), 3T3-L1 adipocytes were incubated in indicated concentrations of BSA, and pretreated with BAY or vehicle for 1 hr, followed by Iso (10 uM) for 3 hr, and mRNA was analyzed for expression of indicated genes by normalizing to % Control (Ctl). Statistical significance is shown for comparisons of Ctl with different BSA concentrations (*** P<0.001; ** P<0.01).

3.2 The induction of PAI-1 is partly mediated by ceramides/sphingolipids

The above results strongly indicate that intracellular FFA or FFA-derived metabolites trigger adipocyte inflammation. To explore the possibility that downstream metabolites might mediate FA-dependent inflammatory cytokines expression, we tested the role of ceramides/sphingolipids, which are potential mediators in adipose tissue inflammation (Kolak et al. 2007, Samad et al. 2006). We first examined whether β-AR stimulation increases the production of ceramides and sphingolipids in 3T3-L1 adipocytes by performing LC-MS/MS quantification. Stimulation of 3T3-L1 cells with isoproterenol elevated the production of various species of ceramides and sphingolipids (Figure 3A). These bioactive signalling lipids can be generated de novo from palmitic acid by the rate limiting enzyme serine palmitoyltransferase (SPT). Thus, to test the potential involvement of de novo ceramides/sphingolipids in the induction of inflammatory cytokines expression, we examined the effects of myriocin, a selective inhibitor of SPT (Miyake et al. 1995). Treatment of 3T3-L1 adipocytes with myriocin significantly reduced the expression of PAI-1 after β-AR activation, but did not affect basal levels of gene expression (Figure 3B). Myriocin did not reduce IL-6 levels, suggesting that de novo ceramides are not involved in the regulation of this cytokine and that alternate pathways are involved (Figure 3C). Treatment with myriocin did not affect lipolysis, demonstrating that the effects of SPT inhibition are not due to alteration in FFA mobilization (Figure 3D). These results indicate that intracellular accumulation of FFAs and subsequent generation of ceramides (and/or sphingolipids) contributes to the induction of PAI-1 by β-AR activation.

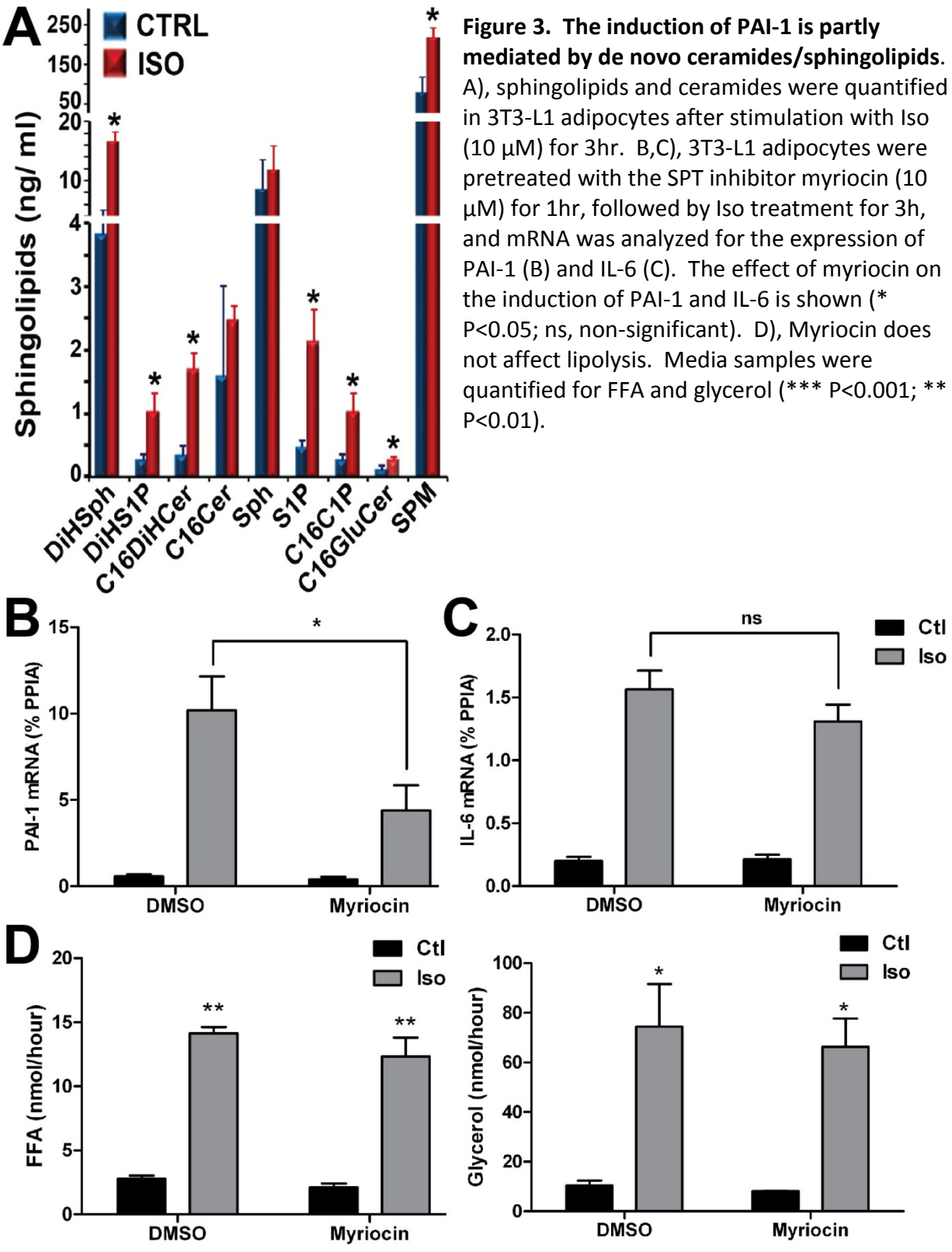

Figure 3. The induction of PAI-1 is partly mediated by de novo ceramides/sphingolipids. A), sphingolipids and ceramides were quantified in 3T3-L1 adipocytes after stimulation with Iso (10 µM) for 3hr. B,C), 3T3-L1 adipocytes were pretreated with the SPT inhibitor myriocin (10 µM) for 1hr, followed by Iso treatment for 3h, and mRNA was analyzed for the expression of PAI-1 (B) and IL-6 (C). The effect of myriocin on the induction of PAI-1 and IL-6 is shown (* P<0.05; ns, non-significant). D), Myriocin does not affect lipolysis. Media samples were quantified for FFA and glycerol (*** P<0.001; ** P<0.01).

3.3 Chapter 3 Discussion

Mounting evidence indicates that disrupting the balance between FA storage and mobilization in adipose tissue contributes to local and systemic inflammation (Arion Kennedy et al. 2009), and is likely to play a role in obesity-induced inflammation (Adilson Guilherme et al. 2008). Central to this is the dysfunctional metabolism of FFAs that leads to the formation of secondary lipid mediators that are causative in insulin resistance (A. Kennedy et al. 2009, Li, Klett, and Coleman 2010). We found that increasing intracellular FFAs enhanced the expression of inflammatory cytokines in adipocytes and this was partly mediated by ceramides. These results support the concept that adipocytes are a source of inflammatory cytokines and that lipid flux in adipocytes toward intracellular FFAs promotes inflammation (Boden 2006, Kolak et al. 2007). Increased ceramides in adipose tissue are associated with fatty liver, greater PAI-1 expression (Kolak et al. 2007), and insulin resistance in humans (Blachnio-Zabielska et al. 2012, Samad et al. 2011). Moreover, altering ceramide metabolism reduces the expression of inflammatory cytokines in adipose tissue and improves whole body glucose tolerance (Mitsutake et al. 2012). Our results indicate that tightly controlling the pool of intracellular FFAs production might be beneficial.

Genetic mouse model that have elevated adipocyte lipolysis suggest that the mobilization of FFAs is not detrimental as long as there is a counter response to upregulate fatty acid oxidation (Cummings et al. 1996, Nishino et al. 2008, Tansey et al. 2001) (See Chapter 5). In agreement, chronic activation of β3-AR enhances the ability to burn lipid, which eventually limits inflammation and improves insulin action (Grujic et al. 1997, Li et al. 2005). On the other hand, enhancing the storage capacity of WAT can also limit the detrimental

effects of FFAs (Kim et al. 2007). In summary work presented in this chapter suggest that preventing the accumulation of intracellular FAs by enhancing lipid oxidation or storage into TG can prevent lipotoxicity in adipocytes.

Chapter 4. Intracellular fatty acids limit the induction of oxidative genes in WAT

4.1 Inhibition of HSL potentiates β3-AR gene expression in WAT

The induction of inflammatory cytokines by β-AR activation in adipocytes is mediated by intracellular FAs (Chapter 3), suggesting that the excessive mobilization of FFAs are detrimental to fat cells and that internal restrains might exist to limit lipolysis. Activation of the β-AR/PKA also promotes the transcription of genes that promote a thermogenic program in WAT such as PGC1α, UCP1 and neuron-derived orphan receptor-1 (NOR-1) (Kumar et al. 2008, Pearen and Muscat 2010). NOR-1 is a PKA-responsive orphan nuclear receptor that is a regulator of UCP1 expression in adipocytes and is highly responsive to β-adrenergic stimulation (Kumar et al. 2008). The objective of this chapter was to explore the relationship between lipolysis, gene transcription and oxidative metabolism in white adipocytes.

The role of lipolysis in regulating the expression of oxidative genes by β3-AR activation was first explored by pharmacological inhibition of HSL. We first examined the effect of lipolysis on the expression of PGC1α and UCP1 and NOR-1 mRNA. As expected, treatment of mice with the β3-AR agonist CL for 3 h elevated the expression of PGC1α, UCP1 and NOR-1 in epididymal WAT. Pretreatment of mice with the HSL inhibitor BAY greatly enhanced CL-mediated expression of PGC1α, UCP1 and NOR-1 by greater than five-fold. Importantly, HSL inhibition did not modify basal levels of gene expression thereby demonstrating an interaction effect of BAY on CL (Figure 4A). We also evaluated whether BAY affected expression of key adipocyte transcription factors, which might account for BAY potentiating the expression of β3-AR target genes. CL elevated expression of CCAAT/enhancer-binding protein β (C/EBPβ), and this effect was not modified by HSL inhibition. CL reduced the expression of PPARγ and BAY did

not have any effect. CL did not modify the expression of PPARα, nor did HSL inhibition. As expected serum FAs and glycerol levels were elevated by β3-AR activation after challenging mice with CL for 45 min (Figure 4B). Levels of both FFA and glycerol were suppressed by HSL inhibition (Figure 4B). There was no effect of BAY on basal lipolysis in the mice. These results suggest that HSL activity limits the expression of oxidative genes in WAT. Of importance is that these genes (PGC1α and UCP1 and NOR-1) are also known direct PKA targets.

To further investigate whether lipolysis limits the expression of oxidative genes in vivo, we evaluated the role of HSL by measuring the expression of PGC1α, UCP1 and NOR-1 in EWAT of HSL-KO mice. Treatment of mice with CL for 6 h upregulated the expression of PGC1α and NOR-1 in wild type mice, and this induction was significantly greater in HSL-KO mice (Figure 4C). The effect of CL on UCP1 expression was not statistical significance (P=0.09) and the effect of BAY on potentiating UCP1 expression was variable (P=0.1762). These results further support the notion that HSL limits the expression of oxidative (PKA-targeted) genes in WAT and argues against any off-target effects for the HSL inhibitor.

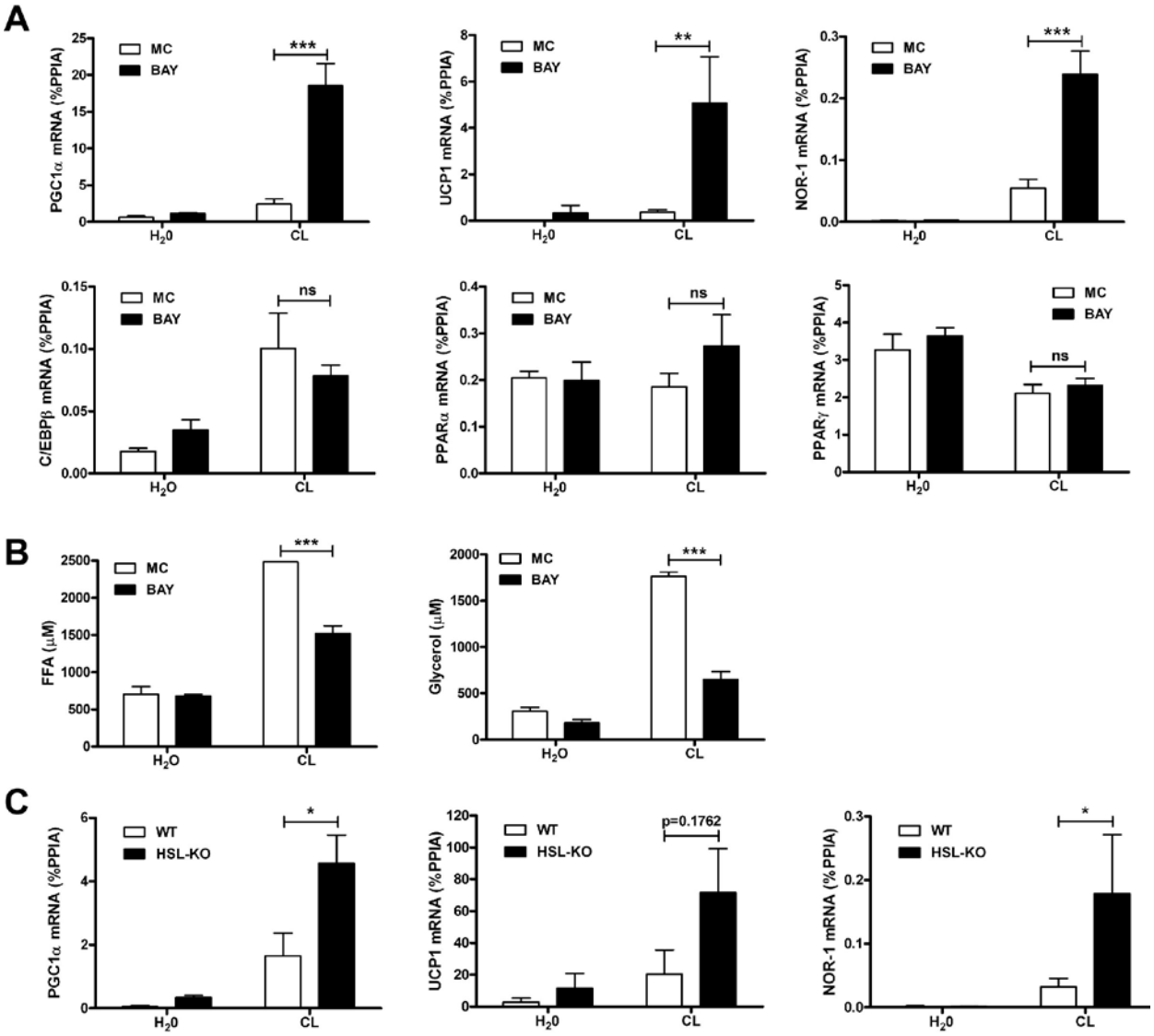

Figure 4. Reducing lipolysis by inhibition or genetic deletion of HSL potentiates PKA-targeted genes during β3-AR activation in white adipose tissue of mice. A) Mice were pretreated with BAY or methylcellulose (MC) for 1h, followed by CL treatment (10 nmol) or vehicle (H_2O) for 3 h and EWAT was collected and analyzed for mRNA expression by qPCR. Data are from 8 mice per group and the effect of BAY is indicated (***=P<0.001; **=P<0.01; ns, non-significant). B) Serum fatty acid and glycerol levels in mice treated with BAY or MC followed by CL (10 nmol) for 45 min. The effect of BAY is indicated (***=P<0.001). C) mRNA levels from EWAT of wild type (WT) and HSL knockout mice (HSL-KO) (n=3-7) were measured by qPCR and normalized to % PPIA. The difference between WT and HSL-KO is indicated (*=P<0.05).

4.2 Limiting lipolysis potentiates β-AR induction of PKA-targeted genes in 3T3-L1 adipocytes

The above experiments suggest that the mobilization of FFAs acutely limit β3-AR signalling in EWAT of mice and as a consequence the expression of oxidative genes. Since adipose tissue consists of numerous cells including adipocytes, adipocyte progenitors, vascular cells and macrophages, 3T3-L1 adipocytes were used as an in vitro cell culture model to test whether lipolysis can directly modify gene expression in a cell autonomous manner. Cultured 3T3-L1 adipocytes were treated with the general β-AR agonist isoproterenol, which elevated the mRNA levels of PGC1α, UCP1 and NOR-1 (Figure 5). HSL inhibition did not have any effect on basal gene expression; however BAY potentiated the induction of PGC1α, UCP1 and NOR-1 by Isoproterenol. These results demonstrate that FA limit β3-AR signalling in a cell autonomous intracellular manner. We further utilized 3T3-L1 adipocytes as a model to explore the relationship between lipolysis and oxidative gene expression.

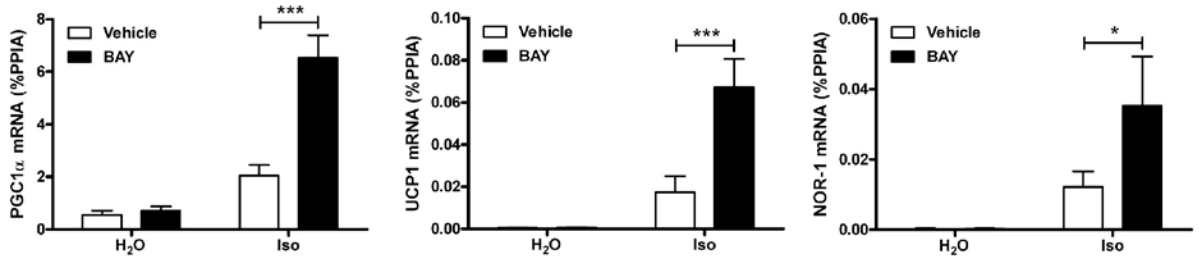

Figure 5. Inhibition of HSL in 3T3-L1 adipocytes potentiates β-AR induction of PKA-targeted genes. A) 3T3-L1 adipocytes were treated with BAY or vehicle (DMSO) followed by 10 µM isoproterenol (Iso) or water (H_2O) for 3 h. Gene expression was measured by qPCR and normalized to % PPIA. Data are from five separate experiments and the effect of BAY is indicated (***=P<0.001; *=P<0.05).

In addition to HSL, adipocytes contain ATGL, the rate limiting enzyme for lipolysis (Eichmann et al. 2012). As HSL is a major DAG lipase (Kraemer and Shen 2002) and rate-limiting for degradation of DAG, this raises the possibility that accumulation of DAG (Haemmerle et al. 2002) might promote PKA-mediated gene expression rather than FA inhibition. Thus, as an additional test that FFAs inhibit β-AR signalling and to eliminate the possibility that DAGs potentiate gene expression, we performed siRNA knockdown of ATGL, the rate limiting enzyme for PKA-mediated DAG production in adipocytes. Treatment of 3T3-L1 adipocyte with ATGL directed siRNA directed against ATGL (siATGL), greatly reduced ATGL protein to almost undetectable levels as indicated by Western blot (Figure 6A). There was no effect on protein levels of HSL, indicating that siRNA treatment did not affect the differentiation of cells (Figure 6A). As expected, knockdown of ATGL reduces basal levels of FFA and glycerol and abolished isoproterenol-induced lipolysis (Figure 6B). There was no effect of ATGL knockdown on basal gene expression, however isoproterenol induction of PGC1α, UCP1 and NOR-1 was greatly potentiated in siATGL treated cells when compared to siCON treated cells (Figure 6C). Overall, these results strongly indicate that FFAs mobilized by either ATGL or HSL limit the induction of PKA-targeted genes by β-AR activation in adipocytes.

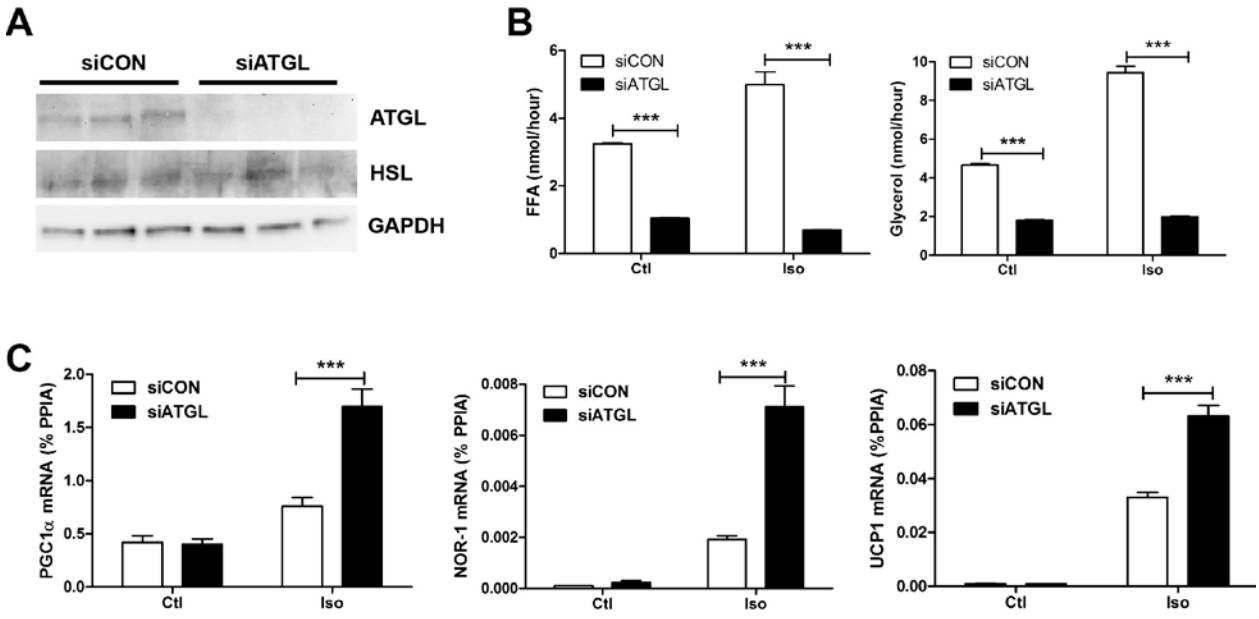

Figure 6. Knockdown of ATGL potentiates β-AR induction of PKA-targeted genes. A) Western blot for ATGL, HSL and GAPDH from three separate wells per siRNA. GAPDH serves as a loading control. B) Glycerol and FFA levels from basal and stimulated (10 µM) siCON and siATGL adipocytes. The effect of ATGL knockdown (siATGL) is indicated (***=P<0.001). C) siCON and siATGL treated adipocytes were stimulated for 3 h with 10 µM isoproterenol (Iso) or water (H_2O). Gene expression was measured by qPCR and normalized to % PPIA. The effect of ATGL knockdown (siATGL) is indicated (***=P<0.001). Data are from 3 experiments performed in triplicate.

4.3 Intracellular FAs reduce β-AR-coupled gene expression in 3T3-L1 adipocytes

The results from Chapter 3 indicate that accumulation of intracellular FAs produces inflammation in white adipocytes. Thus, we next examined whether increasing intracellular FAs could modify the induction of PKA-targets by β-AR activation by blocking FA re-esterification with triacsin C. Triacsin C is an inhibitor of long-chain acyl-CoA synthetases (ACSLs), which control the conversion of FFAs to acyl-CoA derivatives (Coleman et al. 2002). During adrenergic activation FFAs are constantly re-esterified back into TAG (Leibel and Hirsch 1985) by ACSLs. Treatment of cells with triacsin C did not have an effect on basal levels of PGC1α, UCP1 or NOR-1 (Figure 7A), nor did it elevate basal cellular FFA levels (Figure 7B). As expected isoproterenol elevated levels of PGC1α and UCP1, while triacsin C reduced the induction of PGC1α and UCP1 by β-AR (Figure 7A). The expression of NOR-1 was highly variable in these experiments and the effect of triacsin C was not statistically significant. Importantly, triacsin C elevated intracellular FA levels under condition of β-AR activation (Figure 7B). These results suggest that intracellular FFAs limit the induction of PKA targets by β-AR activation. In addition, it argues against the involvement of fatty-acyl-CoAs since their production would be inhibited by triacsin C treatment.

As an addition test, intracellular FAs were increased by limiting efflux by reducing BSA in the medium of cultured 3T3-L1 adipocytes (see Chapter 3, Figure 2). The removal of BSA suppressed the induction of PGC1α and UCP1 gene expression (Figure 7C). HSL inhibition potentiated the induction of PGC1α and UCP1 under all conditions of BSA (Figure 7C). Of note, is that BAY potentiated the expression of PGC1α (4.12 fold) and UCP1 (4.52 fold) the greatest when intracellular FFAs were highest (0% BSA, see Chapter 3, Figure 2).

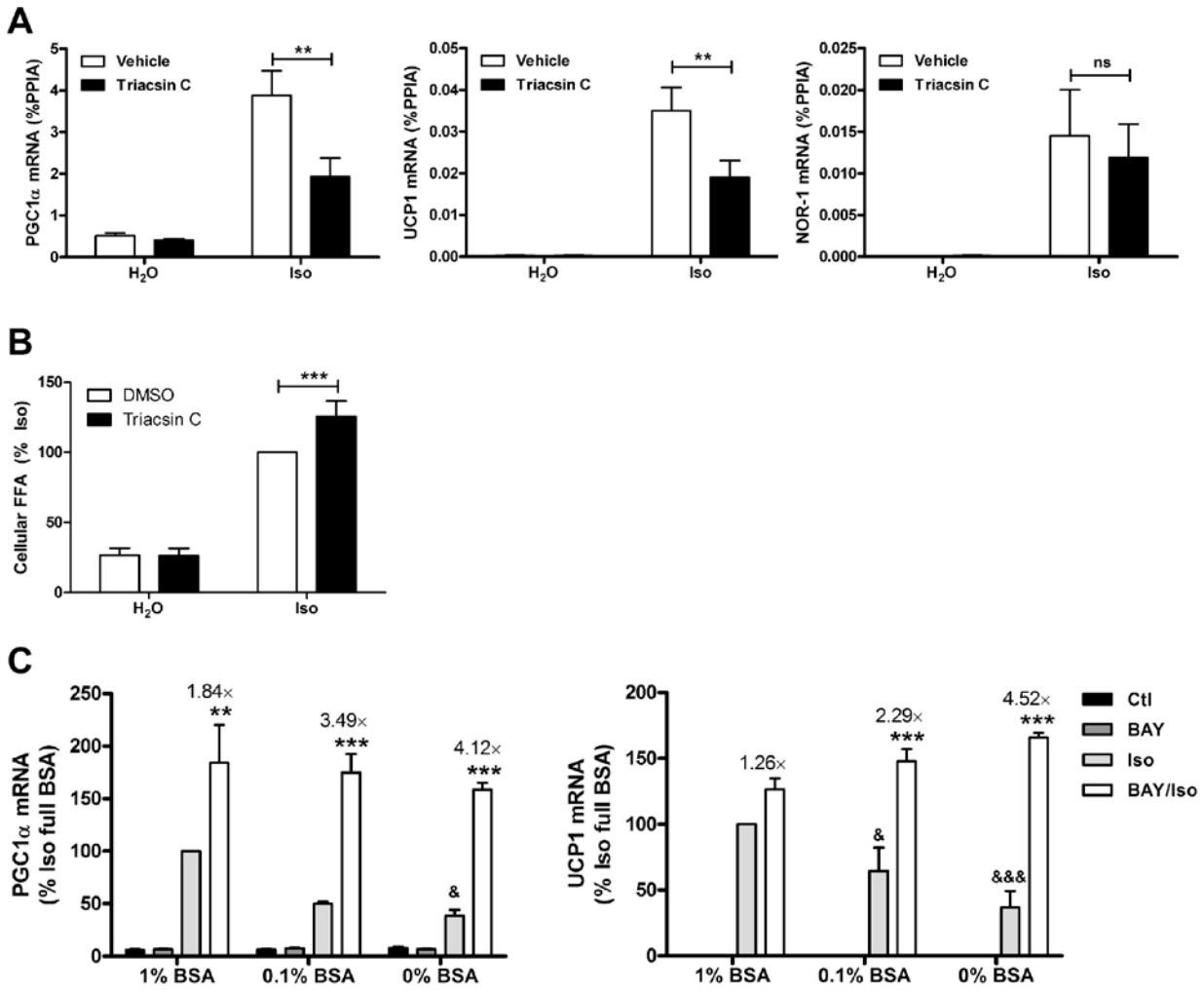

Figure 7. Intracellular FAs suppress the expression of β-AR targeted genes. A) 3T3-L1 adipocytes were treated with 5 µM triacsin C or vehicle (DMSO), followed by 10 µM isoproterenol (Iso) or water (H_2O). Gene expression was measured by qPCR from seven separate experiments and normalized to % PPIA. The effect of triacsin C is indicated (**=P<0.01; ns, non significant). B) 3T3-L1 adipocytes were treated as in A), but Iso treatment was for 1h. Intracellular FA were quantified, and statistical analysis was performed by repeated measures ANOVA to determine the effect of triacsin C (***=P<0.001). C) 3T3-L1 adipocytes were incubated in indicated concentrations of BSA, and treated with BAY (5 µM) or DMSO, followed by Iso (10 µM) for 3 h. mRNA levels were normalized to % PPIA and expressed as % Iso in the presence of 1 % BSA. One-way ANOVA was performed to compare Iso with BAY/Iso at each BSA concentration (**=P<0.01; ***=P<0.001), and to test the effect of Iso at different concentrations of BSA ($^{\&}$=P<0.05; $^{\&\&\&}$=P<0.001). The fold difference between Iso and BAY/Iso is indicated above the bars. Data are from three separate experiments performed in duplicate.

4.4 FAs blunt β-AR induction of cAMP in 3T3-L1 adipocytes by inhibiting adenylyl cyclase

PGC1α, UCP1 and NOR-1 are known cAMP/PKA targets in white adipocytes, so the next logical step was to test whether FA mobilization limits cAMP production. As expected Iso elevated intracellular cAMP levels in 3T3-L1 adipocytes (Figure 8A). Consistent with the effects of BAY on potentiating gene expression, levels of cAMP were further increased by HSL inhibition (Figure 8A). In contrast, treatment of cells with triacsin C, which reduced β-AR-mediated induction of PGC1α and UCP1 (Figure 7A), decreased the production of cAMP by β-AR (Figure 8B).

The above data are consistent with intracellular FFAs limiting the generation of cAMP during β-AR stimulation, and this may explain the effect of HSL inhibition on gene expression. We next wanted to localize the site within the β-AR/PKA cascade by which FA are inhibitory. To accomplish this, we assessed the ability of BAY to potentiate gene expression at the level of AC and at the level of cAMP/PKA. AC was directly activated with FSK and PKA was activated with the cell permeable cAMP analogue 8-Br-cAMP. FSK increased the expression of PGC1α, UCP1 and NOR-1 similar to levels of Iso (Figure 8C). HSL inhibition potentiated the FSK-mediated upregulation of PGC1α and UCP1, which indicates that FFAs inhibit the β-AR cascade, post-receptor. NOR-1 induction by forskolin was variable and the effect of BAY did not reach statistical significance. 8-Br-cAMP elevated the levels of PGC1α, UCP1 and NOR-1 similarly to that of FSK, but in contrast, BAY did not enhance gene expression by 8-Br-cAMP (Figure 8C). These results indicate that FFAs inhibit gene expression upstream of PKA. Together, these results point to FFAs inhibiting β-AR signalling at the level of AC.

We next wanted to interrogate the transcriptional genetic elements by which FFAs might limit expression of PKA-targeted genes. The promoters of the PGC1α, UCP1, and NOR-1 contain various response elements (Cao et al. 2004, Kumar et al. 2009, Rim and Kozak 2002) in addition to cAMP response elements (CREs) that PKA signals through (Reusch, Colton, and Klemm 2000). To explore the role of FFAs on CRE-mediated transcriptional activity in adipocytes, we used a luciferase reporter assay (Himmler, Stratowa, and Czernilofsky 1993). To this end, 3T3-L1 adipocytes were transfected with the CRE luciferase reporter and subsequently treated with FSK. We chose FSK in order attain maximal cAMP/PKA signalling. FSK greatly elevated levels of the CRE luciferase reporter. BAY enhanced reporter activity, while treatment with triacsin C reduced reporter activity (Figure 8D). There was no effect of BAY or triacsin C on basal CRE reporter levels. Importantly, these data are consistent with both the effects of BAY and tracsin C on PKA-mediated gene expression, and cAMP production. Overall these results demonstrate that FFAs limit the expression of PKA-targeted genes by mitigating cAMP/CRE-mediated transcription activity.

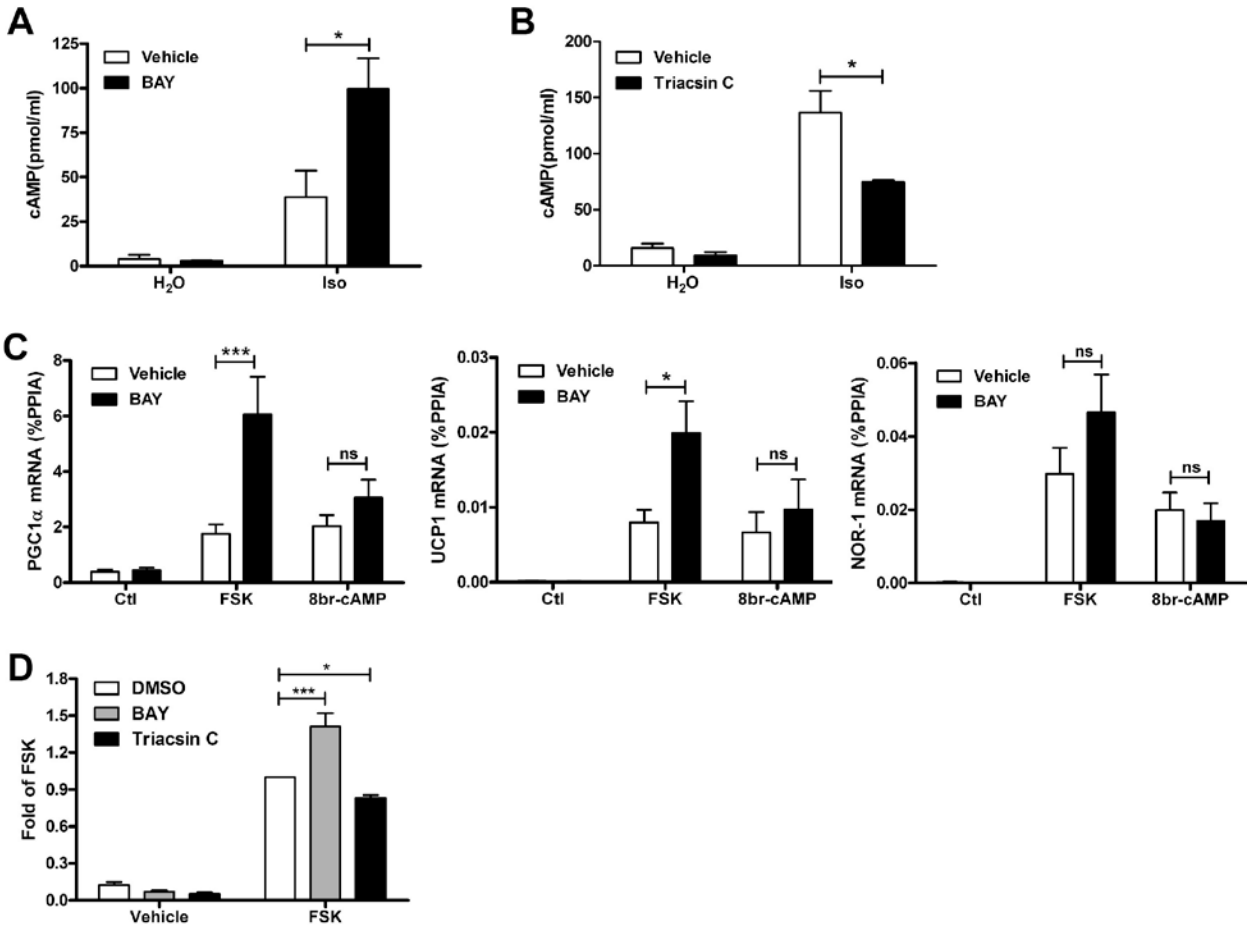

Figure 8. FAs suppress cAMP/PKA signalling at the level of AC. A) 3T3-L1 adipocytes were treated with BAY, followed by Iso for 10 min. cAMP levels were measured from three separate experiments each performed in duplicate. The effect of BAY is indicated (*=P<0.05). B) 3T3-L1 adipocytes were treated with Triacsin C, followed by Iso for 10 min. cAMP levels were measured in two separate experiments each performed in triplicate. The effect of triacsin C is indicated (*=P<0.05). C) 3T3-L1 adipocytes were treated with vehicle (DMSO) or BAY (5 µM) followed by FSK (4 µM) or 8-Br-cAMP (1 mM). mRNA levels were measured by qPCR and normalized to % PPIA. The BAY effect is indicated (***=P<0.001; *=P<0.05; ns, non-significant). D) 3T3-L1 adipocytes, transfected with a CRE reporter, were treated with vehicle (DMSO), BAY or triacsin C, followed by FSK for 6 h. Reporter activity is expressed as fold of FSK and statistical analysis was performed on the normalized data (RLUs/β-gal OD) by repeated measures ANOVA to determine the effect of BAY (***=P<0.001) or triacsin C (*=P<0.05).

4.5 Limiting lipolysis increases mitochondrial activity and promotes CL-induced fat loss

The in vivo (4.1) and in vitro (4.2-4.4) data above indicate that lipolysis restrains cAMP production and thereby limits induction of β-AR/PKA-targeted genes. We wanted to explore the in vivo ramifications of this feedback pathway. We hypothesized that limiting lipolysis could promote the browning of white fat and CL-mediated body fat loss. We choose a period of 5 days of CL treatment, during which the induction of oxidative genes and body fat loss are maximal (Mottillo, Shen, and Granneman 2007). Mice were treated for 5 consecutive days with CL or CL with HSL inhibition (BAY). As we have previously shown (Granneman et al. 2005, Li et al. 2005), CL greatly elevated the expression of long chain acyl-CoA dehydrogenase (LCAD), a marker of fat oxidation; the expression of cytochrome c oxidase subunit VIIIb (Cox8b), a mitochondrial enzyme; and brown adipocyte markers UCP1 and elongation of very long chain fatty acids-like 3 (Elovl3). Mice that were treated with BAY and CL had significantly greater mRNA levels of LCAD, Cox8b and Elovl3, and levels trended to be greater for UCP1 (Figure 9A). We also examined mitochondrial electron transport chain activity in adipose tissue explants. CL tended to increase mitochondrial activity as measured by the reduction of TTC; however, this effect was significant only within the BAY group (Figure 9B). Importantly we also measured body fat in mice. Similarly, 5 days of CL treatment significantly reduced body fat only in mice that had been treated with both CL and BAY (Figure 9C). There was no change in total body weight of mice (Figure 9C). These results indicate that inhibiting HSL enhances the fat loss effects of CL, which is likely driven by the increased browning effects that we observed in WAT.

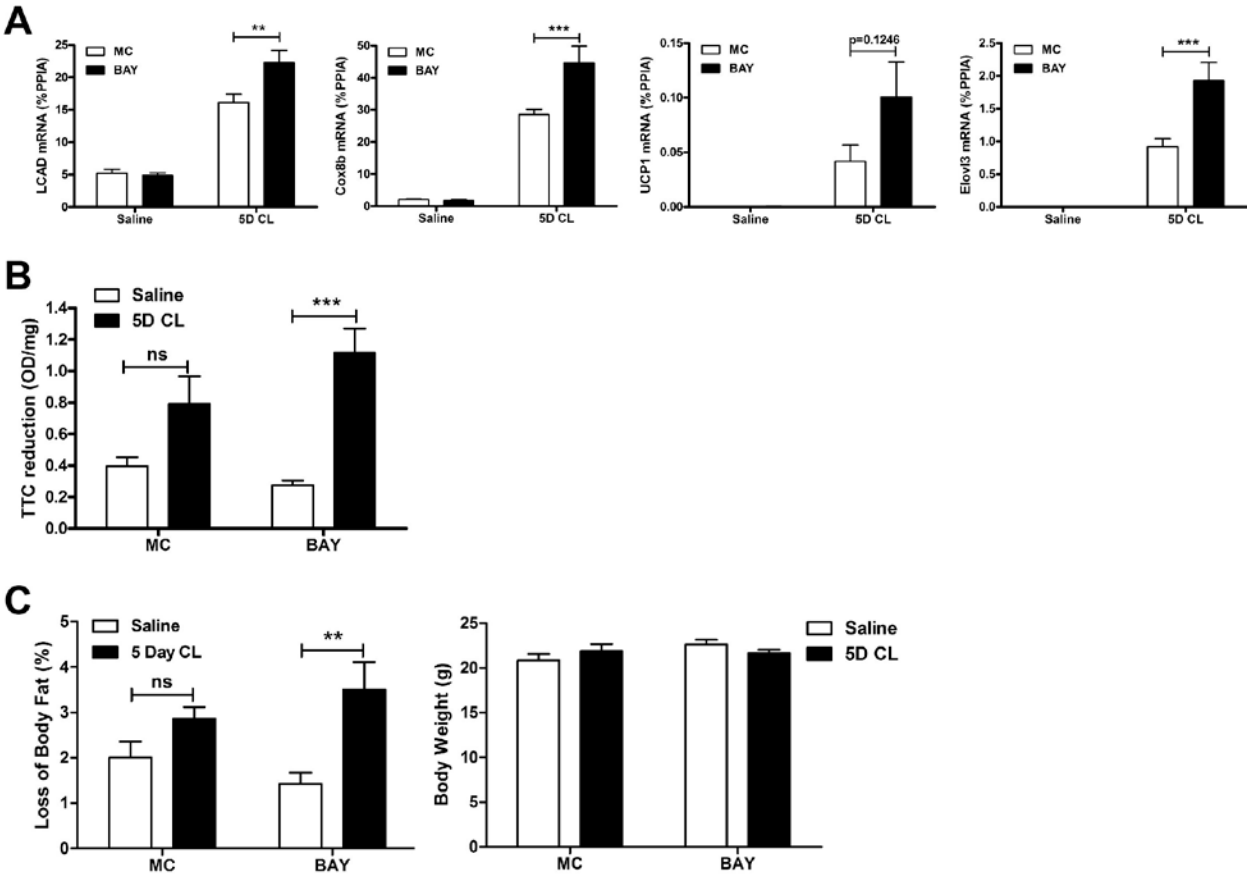

Figure 9. Partial inhibition of lipolysis enhances markers of white fat browning and mitochondrial activity, and promotes fat loss in mice. Mice (n=8 per group) were treated with BAY (30 mg/kg) or methylcellulose (MC) followed by CL (5D CL, 10 nmol) or saline repeatedly for five days. A) mRNA levels in EWAT were measured by qPCR and normalized to % PPIA. The effect of BAY on 5 day CL is shown (***=P<0.001; **=P<0.01). B) TTC reduction as a measure of mitochondrial electron transport chain activity. The effect of 5D CL treatment is shown (***=P<0.001; ns, non significant). C) Body fat composition was measured in mice by MRI and expressed as loss of body fat. The effect of 5D CL is indicated (**=P<0.01; ns, non-significant).

4.6 Chapter 4 Discussion

The current results imply a negative feedback system whereby FAs limit cAMP production at the level of AC. As a consequence, this feedback system limits the induction of oxidative (PKA-target genes) in WAT (Figure 10A). FAs suppress cAMP production in cultured adipocytes and in adipocyte membranes (Fain and Shepherd 1975, Ho et al. 1975); however, the impact of this potential feedback on PKA-mediated gene expression and the in vivo effects of β3-AR activation are not known. Our data indicate that FFAs limit cAMP production at levels of β3-AR occupancy that are sub-maximal with respect to the induction of PKA-targeted genes. Thus, inhibiting HSL relieves the negative feedback on AC, which allows for greater cAMP production and increased expression of oxidative genes such as PGC1α and UCP1 (Figure 10B). Inhibition of HSL dampens the relationship between cAMP production and lipolysis without affecting the relationship between cAMP and oxidative gene expression, and thus provides a better balance between FA mobilization and oxidation. Consequently, inhibition of HSL enhanced mitochondrial activity and weight loss associated with β3-AR agonist treatment. While it seems contradictory that inhibition of HSL would promote greater oxidation and fat loss, this can be explained by the presence and activation of ATGL. Of note, is that the EWAT of HSL-KO mice attain a brown adipose tissue phenotype and are resistant to diet-induced obesity (Strom et al. 2008). In a similar fashion, overexpression of ATGL in adipose tissue promotes increased oxidative metabolism and greater weight loss in mice (Ahmadian et al. 2009).

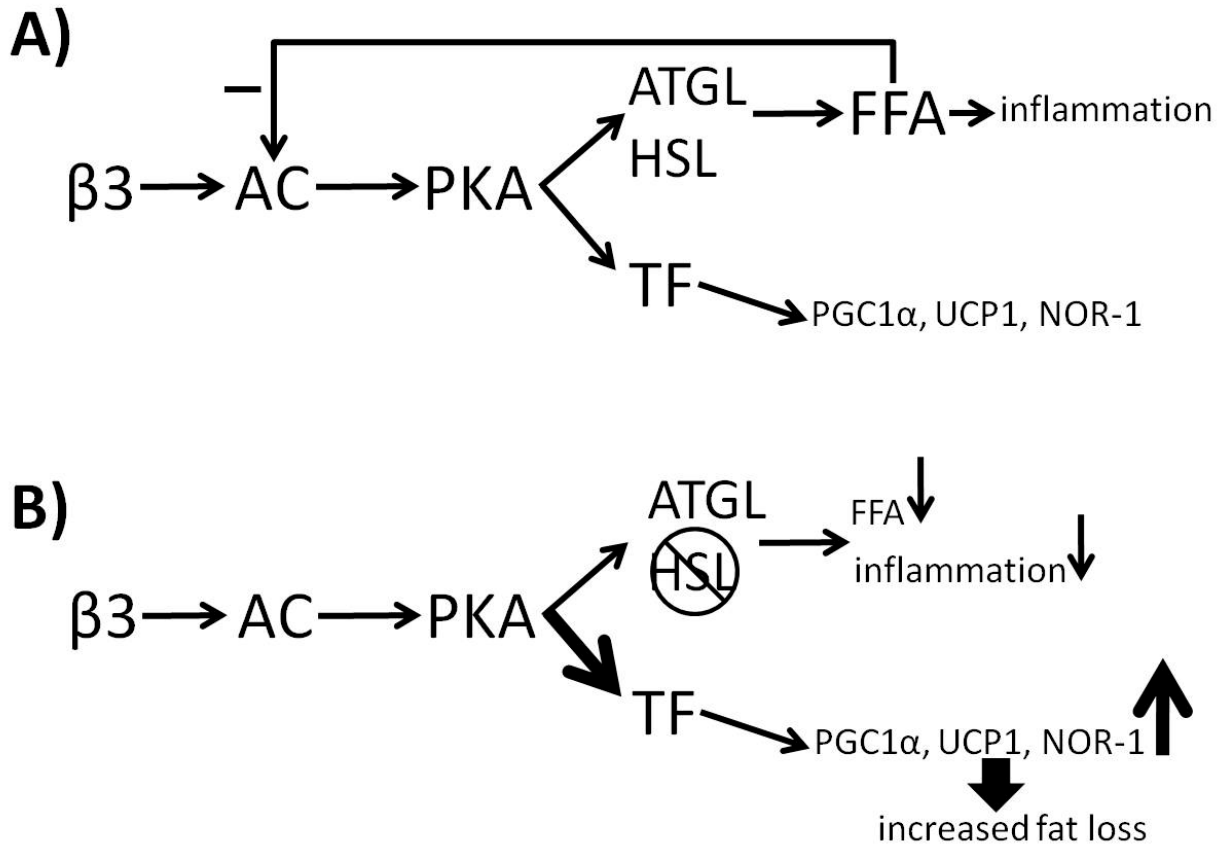

Figure 10. Limiting lipolysis promotes β3-AR signalling in WAT. A) Acute stimulation of the β3-AR activates adenylyl cyclase (AC) and PKA and subsequently ATGL and HSL, and transcription factors (TF) which promote the transcription of genes such as PCG1α, UCP1 and NOR-1. The mobilization of FAs causes inflammation and feedback to reduce AC activity. B) During inhibition of HSL in vivo, FA mobilization is reduced, inflammation is suppressed and the negative feedback on AC is relieved, thereby increasing expression of PKA-targeted genes and promoting fat loss.

Metabolic remodelling of WAT by β3-AR is a dynamic process, and it is likely that limiting lipolysis would facilitate the early, but not the late phases of remodelling (Granneman et al. 2005, Li et al. 2005). In the early phase of β3-AR activation, adipose tissue has low capability for lipid oxidation, and the mobilization of FAs triggers inflammation (Chapter 3), and suppresses cAMP production. By limiting FAs during acute β3-AR activation, inflammation is prevented (Chapter 3) and the transition to an oxidative phenotype is facilitated. In the late phase, the capacity of WAT for lipid oxidation is enhanced, and FAs are not expected to promote inflammation or limit PKA-targeted gene expression. In agreement, we have previously shown that the deleterious effects of mobilized FAs are mitigated once the oxidative capacity of WAT has been expanded (Li et al. 2005). Thus, once WAT has been fully "browned", lipolysis could be fully reinstated to permit greater fat loss. In this case lipolysis in WAT would be greater coupled to oxidation, similarly to that of BAT (Chapter 5).

Chapter 5. Lipolytic products activate PPARα and δ in brown adipocytes to match fatty acid oxidation with supply

5.1 Lipolysis is required for the maximal induction of thermogenic gene expression by β-AR in brown adipocytes and BAT

FFAs are necessary and sufficient to drive thermogenesis in BAT. Based upon the role of lipolysis in activating thermogenesis in brown fat and our previous work that lipolysis produces inflammation and restricts cAMP generation in white adipocytes, we suspected that lipolysis would have the opposite effects in modulating the expression of oxidative genes in BAT. We initially tested the role of lipolysis in promoting gene expression in BAT by pharmacological inhibition of HSL. Mice were first challenged with the β3-AR agonist CL 316,243 (CL) and the expression of thermogenic genes in BAT was analyzed by qPCR. CL induced the expression of PGC1α, PPARα, pyruvate dehydrogenase kinase 4 (PDK4), UCP1 and NOR-1 in BAT (Figure 11A), while inhibition of HSL with BAY reduced the induction of PGC1α, PPARα, PDK4 and UCP1, but not NOR-1. As an additional test of the in vivo role of HSL, we challenged WT/Het and HSL-KO mice with CL. CL induced the expression of PGC1α, PPARα, PDK4, UCP1 and NOR-1 in WT/Het mice and this effect was reduced in HSL-KO mice for PGC1α and UCP1 and trended to for PPARα (P=0.058) and PDK4 (P=0.18) (Figure 11B). The induction of NOR-1 was similar between WT/Het and HSL-KO mice. These results are in complete contrast to what we observed in white fat, where HSL inhibition potentiated the expression of genes by β3-AR (Chapter 4). Importantly, this suggests that different mechanisms are at work in modulating the induction of genes by B3-AR in WAT vs. BAT.

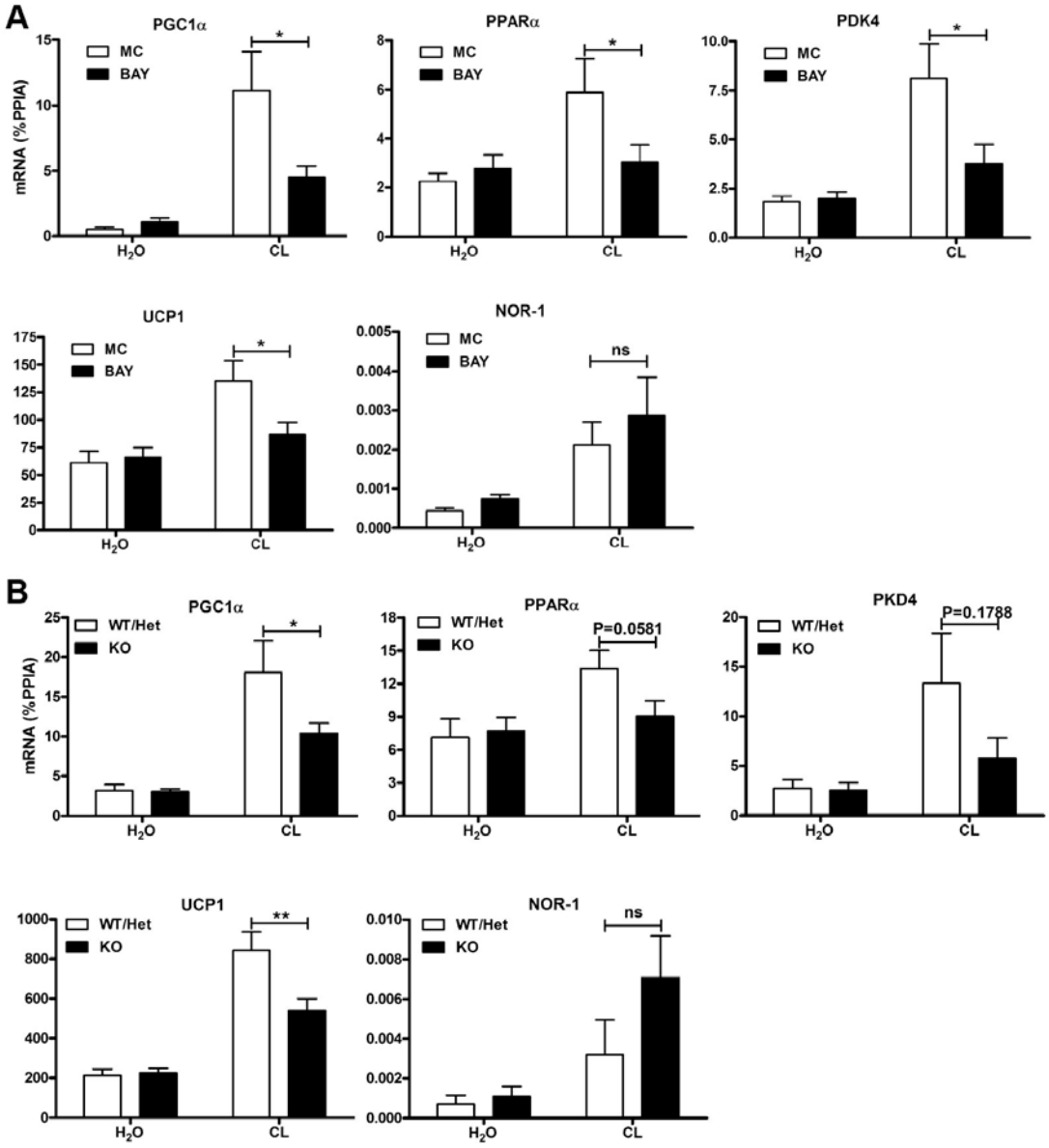

Figure 11. HSL is required for β3-AR mediated induction of thermogenic genes in BAT. *A*, mice (n=11) were pretreated with BAY (30 mg/kg) or methylcellulose (*MC*) for 1 h, followed by CL-316,243 (*CL*; 10 nmol) or vehicle (*H_2O*) for 3 h and BAT was analyzed for mRNA expression by QPCR and normalized to % PPIA. *B*, mice (n=13) *WT*, heterozygous (*Het*), or deficient (*KO*) for HSL were treated with CL (10 nmol) for 6 h and BAT was analyzed for mRNA as above. The effect of BAY (two-way ANOVA), or difference between WT/Het and HSL-KO mice (two-way ANOVA) is indicated (**P < 0.01; *P < 0.05; n.s., non-significant).

We next wanted to verify our in vivo finding in a cell culture model by using an immortalized brown adipocyte cell line. Treatment of BAs with the β-AR agonist Iso similarly induced the expression of PGC1α, PPARα, PDK4, and NOR-1 (Figure 12A). HSL inhibition (BAY) did not have any effect on the basal levels of gene expression; however BAY reduced the induction of PGC1α, PPARα, PDK4, but not NOR-1 by β-AR activation. As expected, lipolysis was increased by β-AR, and reduced by BAY (Figure 12B). To ensure that these effects on gene expression were not due to alterations at the level of the β-AR, we bypassed the β-AR by using the cAMP analogue, 8-Br-cAMP. Stimulation of BAs with 8-Br-cAMP induced the expression of PGC1α, PPARα, PDK4, and UCP1, and this effect was reduced by BAY (Figure 12C). Inhibition of HSL did not have any effect on the induction of NOR-1 mRNA by 8-Br-cAMP. These results suggest that HSL functions to promote the induction of a subset of genes by β-AR/PKA activation in BAs. Importantly, many of the genes that are regulated in a HSL-dependent manner are important in BAT thermogenesis.

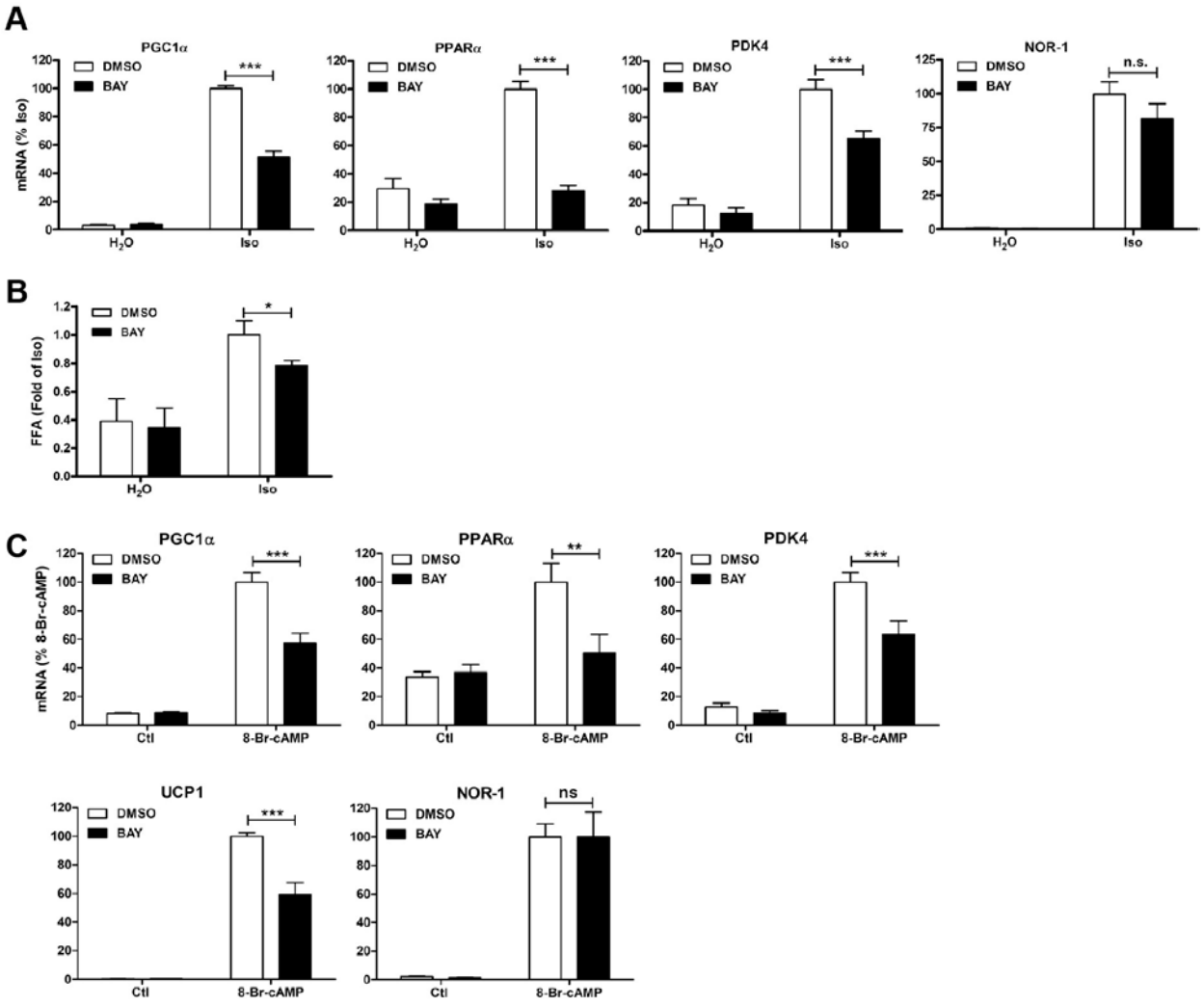

Figure 12. HSL is required for β-AR mediated induction of oxidative genes in brown adipocytes. *A*, brown adipocytes were treated with BAY (5 μM) or vehicle (DMSO), followed by isoproterenol (*Iso*; 10 μM) for 4 h. mRNA levels were measured by QPCR, normalized to % PPIA and expressed as a % Iso. *B*, *FFA* in the media were measured after 4 h and expressed as a fold of Iso. *C*, brown adipocytes were treated as in *A*, except cells were stimulated with 8-Br-cAMP (1 mM). Data are from 3-4 independent experiments performed in duplicate and analyzed by two-way ANOVA to determine the effect of BAY (***P < 0.001; **P < 0.01; *P < 0.05; n.s., non-significant).

Adipocytes also contain ATGL, which is the rate limiting enzyme for lipolysis. We tested the role of ATGL in the upregulation of thermogenic genes by β-AR activation by making stable cell lines that express a control shRNA (shCON) or shRNA against ATGL (shATGL). Brown adipocyte cells expressing shATGL demonstrated reduced protein levels of ATGL (Figure 13A), and a reduction in lipolysis under basal conditions and also with β-AR activation (Figure 13B). Importantly, there was no observable difference in the ability of cells to differentiate as determined by bright field microscopy (Figure 13C). Treatment of shCON cells with Iso promoted the expression of PGC1α, PPARα and PDK4, and this induction was lower in shATGL cells (Figure 13D). The upregulation of NOR-1 by Iso was similar between shCON and shATGL cells. Inhibition of HSL further diminished lipolysis in shATGL cells (Figure 13B) and resulted in a further reduction is the expression of PGC1α and PPARα (Figure 13D). The effect of HSL inhibition on PDK4 mRNA in shATGL cells did not have any further effect as levels of PDK4 were already greatly reduced (Figure 13D). BAY did not affect the expression of NOR-1. Together, these results indicate that lipolysis via the concerted action of ATGL and HSL regulates the expression of thermogenic genes by β-AR activation in BAs.

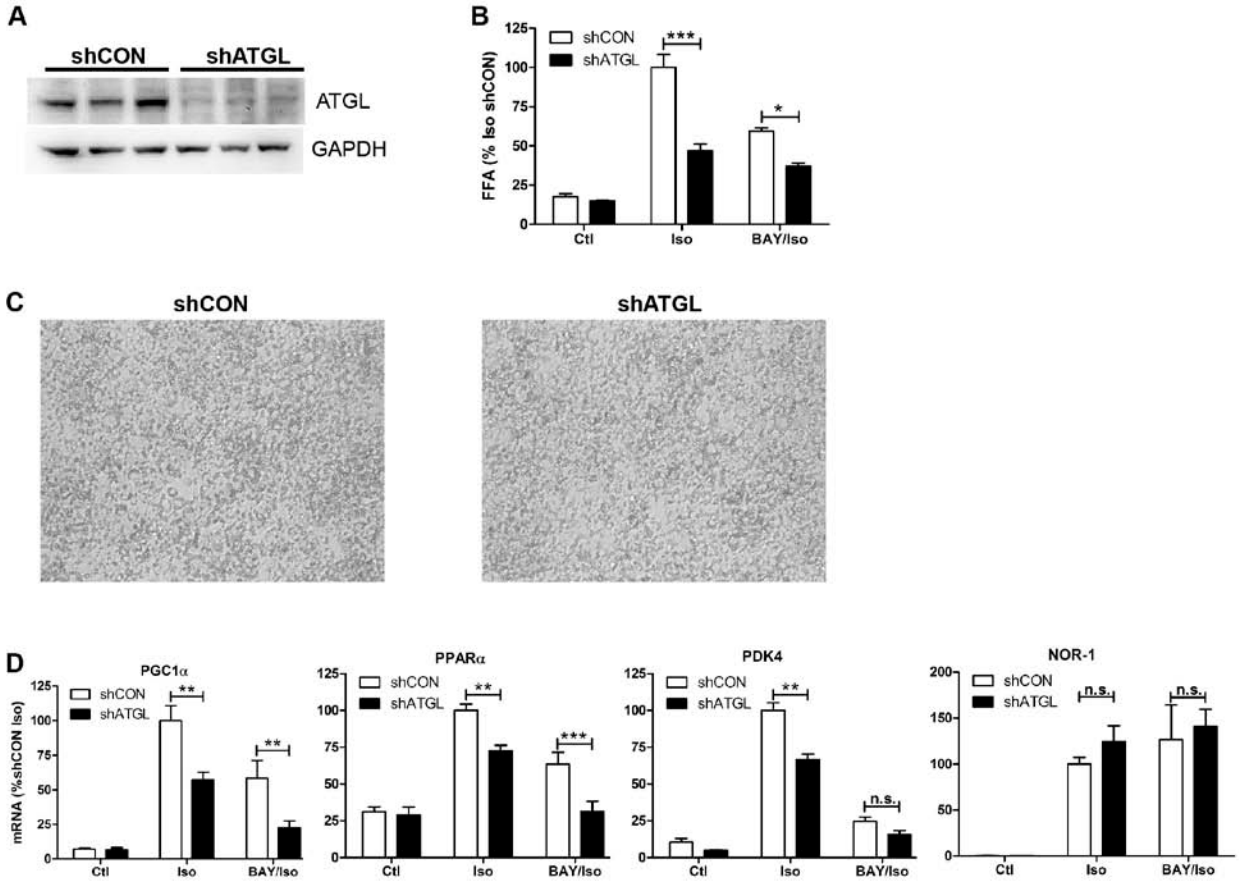

Figure 13. Knockdown of ATGL reduces the induction of oxidative genes by β-AR activation in brown adipocytes. *A*, western blot was performed on shCON and shATGL brown adipocytes for ATGL and GAPDH. *B*, *FFA* in the media were measured after 4 h and expressed as a fold of Iso. *C*, brightfield images of differentiated shCON and shATLG brown adipocytes. *D*, shCON and shATGL brown adipocytes were treated with *Iso* (10 µM) or BAY and Iso (*BAY/Iso)* for 4 h and mRNA levels were measured by QPCR, normalized to %PPIA and expressed as a percent of shCON Iso. Data are from 3 separate experiments performed in duplicate, and the difference between shCON and shATGL cells by two-way ANOVA is indicated (***P < 0.001; n.s., non-significant).

5.2 Increasing fatty acids promotes thermogenic gene transcription in brown adipocytes

The above results suggest that lipolytic products, which are likely FFAs, function to positively regulate gene transcription in BAs. To further test the role of FFAs we performed experiments to manipulate endogenous FA levels. First, fatty acids levels were modified by blocking their oxidation via inhibition of carnitine palmitoyl-transferase 1 (CPT1), which is the rate limiting step for entry of activated FAs into the mitochondria. Inhibition of CPT1 by etomoxir in BA had no effect on the basal expression levels of PGC1α, PDK4, PPARα and UCP1. However, etomoxir enhanced the induction of PGC1α, PDK4, PPARα and UCP1 by Iso (Figure 14A). Etomoxir did not have any effect on efflux of FFAs into the media (Figure 14B). Secondly, FFA levels were increased in brown adipocyte by inhibiting the activity of long chain acyl-CoA synthetases (ACSLs) with Triacsin C. ACSLs are required to catalyze the formation of fatty acyl-CoA from FFAs. Triacsin C elevated FFA levels (Figure 14C), which was sufficient to promote the expression of PGC1α, PDK4, UCP1 and PPARα in the absence of PKA activation (Figure 14D). These results demonstrate that endogenous FAs promote the expression of thermogenic genes in BAs.

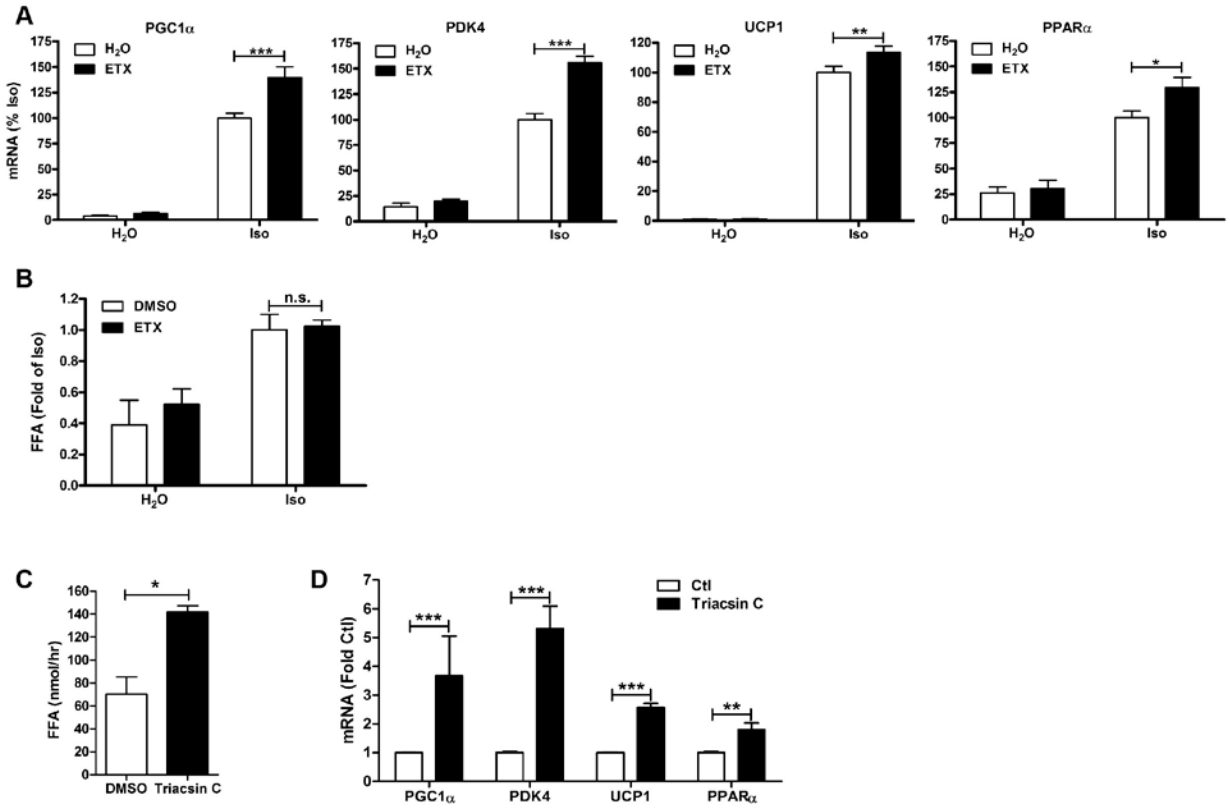

Figure 14. Endogenous fatty acids increase the transcription of genes involved in thermogenesis in brown adipocytes. *A*, brown adipocytes were treated with etomoxir (*ETX;* 50 µM) or vehicle (*H_2O*) followed by *Iso* (10 µM) for 4 h. mRNA levels were measured by QPCR, normalized to %PPIA and expressed as a percent of Iso. *B*, brown adipocytes were treated with *triacsin C* or vehicle (*DMSO*) and *FFA* in the media were measured after 4 h and expressed as nmol/hr. *C*, brown adipocytes were treated as in *B*, and mRNA levels were quantified by QPCR and expressed as a fold of control (*Ctl*). Data are from 3-4 separate experiments performed in duplicate, and the effect of ETX or triacsin C is shown (***P < 0.001; **P < 0.01; *P < 0.05).

5.3 PPARα and PPARδ mediate the induction of thermogenic genes by β-AR activation

We next addressed the mechanisms of how lipolytic products increased thermogenic gene expression in BAs. Common among the promoter regions of the PGC1α, PDK4 and UCP1 genes are binding sites for the family of nuclear receptor called PPARs, which consists of PPARα, PPARδ and PPARγ. The PPARs regulate various aspect of lipid metabolism and are known to be activated by various lipid species. First we tested the role of PPARs in regulating the expression of thermogenic genes by β-AR by using selective antagonists (Figure 15A,C). Treatment of BAs with antagonists against PPARα and δ significantly reduced the induction of gene expression (Figure 15A). The PPARα antagonist GW6471 reduced the induction of PGC1α, PPARα, PDK4 and UCP1, while antagonism of PPARδ by GSK0660, reduced the upregulation of PPARα, PDK4 and UCP1 and not PGC1α. Neither the PPARα nor PPARδ antagonists had any effect on lipolysis (Figure 15B). In contrast, the effect of PPARα and δ antagonists, there was no effect of the PPARγ antagonist GW9662 on the induction of genes by β-AR agonism (Figure 15C).

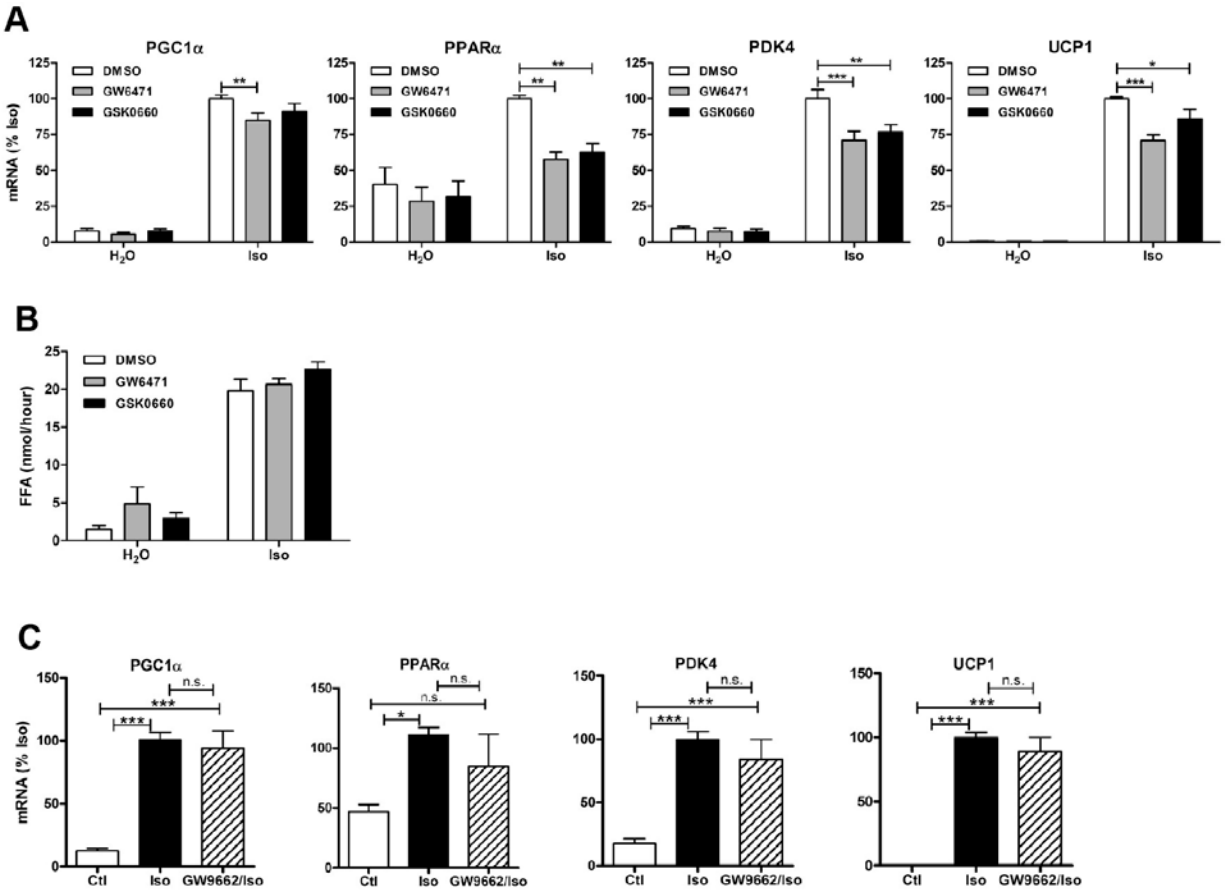

Figure 15. Antagonists against PPARα and δ, but not PPARγ reduce the induction of thermogenic genes by β-AR activation in brown adipocytes. *A*, brown adipocytes were treated with antagonists against PPARα (*GW6741*; 10 μM) or PPARδ (*GSK0660*; 2 μM) followed by *Iso* (10 μM) for 4 h. mRNA levels were measured by qPCR, normalized to %PPIA and expressed as a percent of Iso. Data are from 4 separate experiments performed in duplicate, and the effect of GW6471 or GSK0660 by two-way ANOVA is shown (***P < 0.001; **P < 0.01; *P < 0.05). *B*, FFA levels from *A* were measured in the medium and expressed as nmol/hr. *C,* brown adipocytes were treated with PPARγ antagonist (GW9662, 30 μM) followed by Iso for 4 h. The differences between groups were evaluated by one-way ANOVA
.

As pharmacological probes can have disadvantages with selectivity, we wanted to further test the above results by performing siRNA knockdown of PPARα and PPARδ. Targeted siRNAs effectively knocked down mRNA levels of PPARα and δ by greater than 60% (Figure 16A). Knockdown did not affect basal or Iso stimulated lipolysis as detect by efflux of FFAs into the media (Figure 16B). Knockdown of PPARα (siPPARα) significantly reduced the induction of PGC1α, PDK4, and UCP1 by Iso, but not NOR-1. siRNA against PPARδ (siPPARδ) reduced the upregulation of PDK4 and UCP1 by isoproterenol (Figure 16C). PPARδ knockdown did not have any effect on the induction of PGC1α or NOR-1 mRNA by isoproterenol. Importantly, that knockdown of PPARα or PPARδ did not have any effect on lipolysis or the induction of NOR-1 mRNA, suggests that siRNA treatment did not have any effect on the differentiation of BAs. In conjunction with the above antagonist studies, these results demonstrate that PPARα and δ are required for the maximal induction of thermogenic genes by β-AR activation.

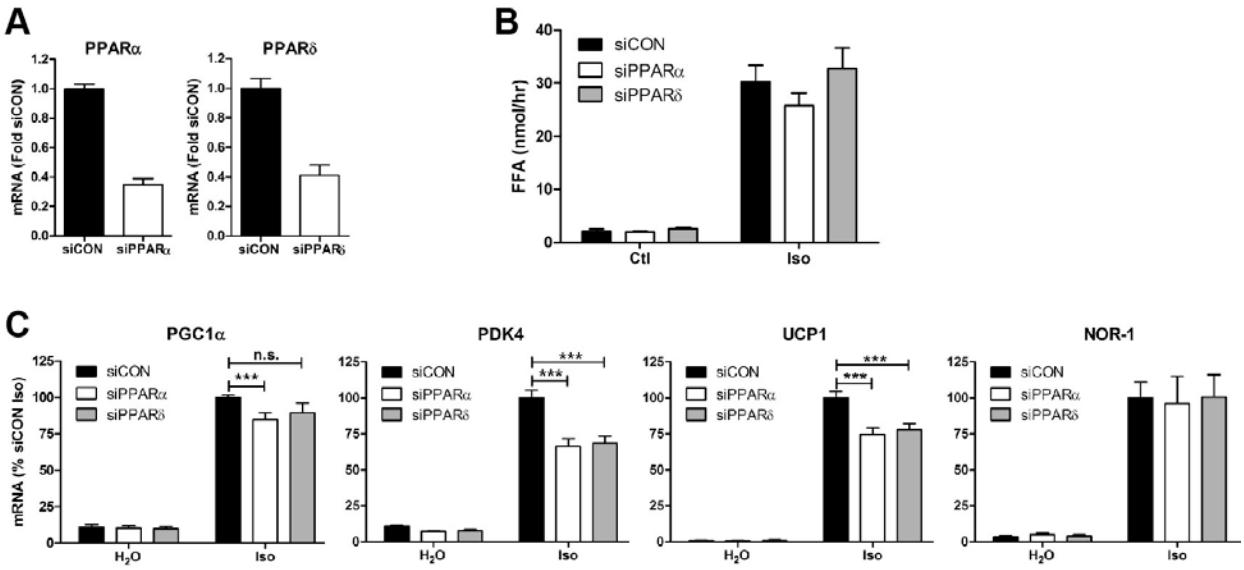

Figure 16. Knockdown of PPARα and δ reduces the induction of thermogenic genes by β-AR activation in brown adipocytes. *A*, mRNA levels of PPARα and δ in brown adipocytes treated with control (siCON), PPARα (siPPARα), or PPARδ (siPPARδ) siRNA normalized to %PPIA and expressed as a Fold of siCON. *B*, FFA levels in the medium from *C* were measured at 1 h after stimulation and expressed as nmol/hr. *C,* siCON, siPPARα or siPPARδ treated brown adipocytes were stimulated with Iso for 4 h, mRNA was quantified and expressed as a percent of siCON Iso. Data are from 3 separate experiments performed in triplicate and the effect of siPPARα or siPPARδ by two-way ANOVA is shown (***P < 0.001; n.s., non significant). *C*,

5.4 Lipid droplets generate ligands that can transcriptionally activate PPAR α and δ, but not PPARγ

The above results suggest that PPARα and δ detect lipolytic products. Thus, we investigated whether lipolysis produced agonists for PPARs. PPARs are activated when agonists enter the nucleus, bind their cognate receptor, and recruit co-activator molecules. While the mechanisms of nuclear receptor activation are well known, the pathways that produce ligands and their site of production are poorly understood. We tested whether lipid droplets generate ligands for PPARs by developing a fluorescent reporter assay that is based upon the ligand-dependent interaction between PPARs and the transcriptional co-activator SRC-1. We fused

the ligand binding domain (LBD) of PPARs to the lipid droplet protein Plin1, and the LxxLL domain of SRC-1 to EYFP (Figure 17A). Therefore, if PPAR ligands are generated by lipolysis, they would be detected by translocation of EYFP-SRC1 from the cytoplasm to the Plin1-PPAR LBD fusion on the lipid droplet. In BAs co-transfected with the Plin1-PPARα fusion and EYFP-SRC1, EYFP fluorescence was predominantly cytosolic under basal conditions (Figure 17B). EYFP-SRC1 translocated to the lipid droplet surface within minutes after addition of 8-Br-cAMP, and the intensity of fluorescence continued to increase for 20 min (Figure 17B, arrows). HSL inhibition greatly reduced the recruitment of EYFP-SRC1 to lipid droplets (Figure 17B, arrows). As a measure that the PPARα fluorescent reporter could be rescued with HSL inhibition, the PPARα agonist, Wy 14,263, was added to fully stimulate reporter translocation to lipid droplets (Figure 17B; Wy). By normalizing the data to the maximal effect induced by Wy, there was a significant effect of HSL inhibition on the activation of the PPARα reporter (Figure 17C). Similarly, stimulation of BAs with 8-Br-cAMP increased the activity of the PPARδ reporter, and this effect was nearly abolished by BAY (Figure 17D). In contrast, 8-Br-cAMP did not promote EYFP-SRC1 translocation to lipid droplet with PPARγ, but the reporter was fully activated with the PPARγ ligand rosiglitazone (Figure 17E). We also found that activation of the PPARα fluorescent reporter was lower in BAs with ATGL knockdown (Figure 17F). These results suggest that ligands for PPARα and δ are generated by lipid droplets in response to lipolysis.

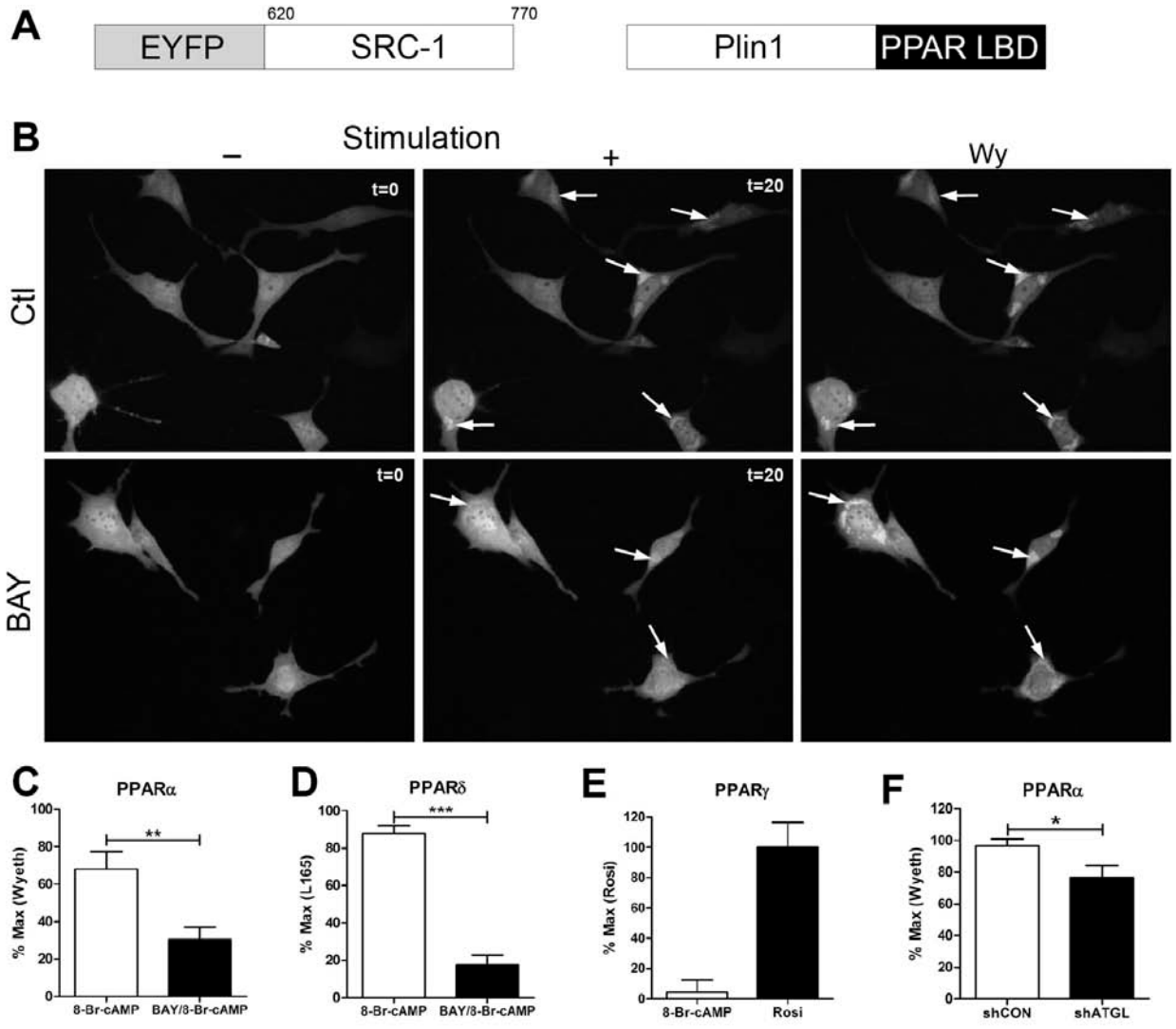

Figure 17. Ligands for PPARα and δ, but not PPARγ are created at the lipid droplet surface in response to lipolysis. *A*, schematic representation of constructs used for the fluorescent reporter assays (amino acids for SRC-1 are shown). *B,* brown adipocytes transfected with a PPARα reporter were pretreated with DMSO (*Ctl*) or BAY (5 μM) for 10 min. Representative images shown prior to stimulation (-, t=0) with 1 mM 8-Br-cAMP, or after 20 min of stimulation (+, t=20), and after addition of PPARα ligand (*Wy* 100 μM). *C*, the PPARα reporter was quantified by normalizing the region of interest (ROI) after treatment with 8-Br-cAMP or BAY/8-Br-cAMP to the maximal effect of *Wyeth* from 2-3 coverslips per experiments (n=3). *D,* The PPARδ reporter was normalized to the maximal effect of L-165,041 (*L165*, 10 μM). The effect of BAY was determine by unpaired t-test (***P < 0.001; **P < 0.01). *E*, the PPARγ reporter was quantified by normalizing the ROI after 8-Br-cAMP to the maximal effect of *Rosi* (10 μM). *F*, the PPARα reporter was quantified in shCON and shATGL brown adipocytes after treatment with 8-Br-cAMP as above, and the difference was determined by unpaired t-test (*P < 0.05).

To verify that activated PPAR reporters were correctly targeted to lipid droplets, cells were co-stained with a fluorescent neutral lipid dye, lipidTOX, and imaged for YFP fluorescence (Figure 18). Arrows note where activated PPAR reporters localize to lipid droplets in the same cells.

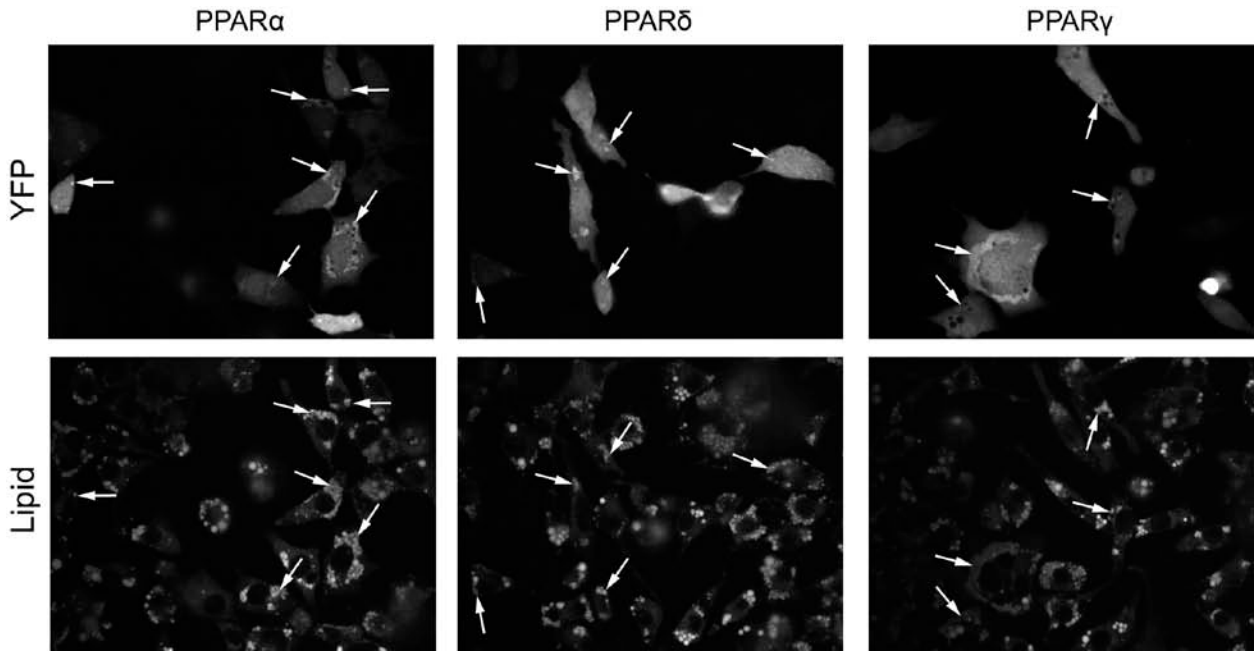

Figure 18. PPAR fluorescent reporters are correctly targeting to lipid droplets. Brown adipocytes transfected with the different PPAR reporters were treated with respective PPARs ligands, stained with LipidTOX and imaged for EYFP flouresecence (YFP) and neutral lipids (TGs).

To further establish that FAs are ligands for PPARα and δ, the addition of exogenous FAs was tested. Addition of oleic acid (OA), which is a major FA found in TGs, was sufficient to activate PPARα and δ, but not γ, while addition of ligand fully activated the three reporters (Figure 19A). Quantification of these results relative to intrinsic activity of ligand demonstrates that 8-Br-cAMP activated PPARα and δ nearly as much exogenous OA (80%; Figure 19B).

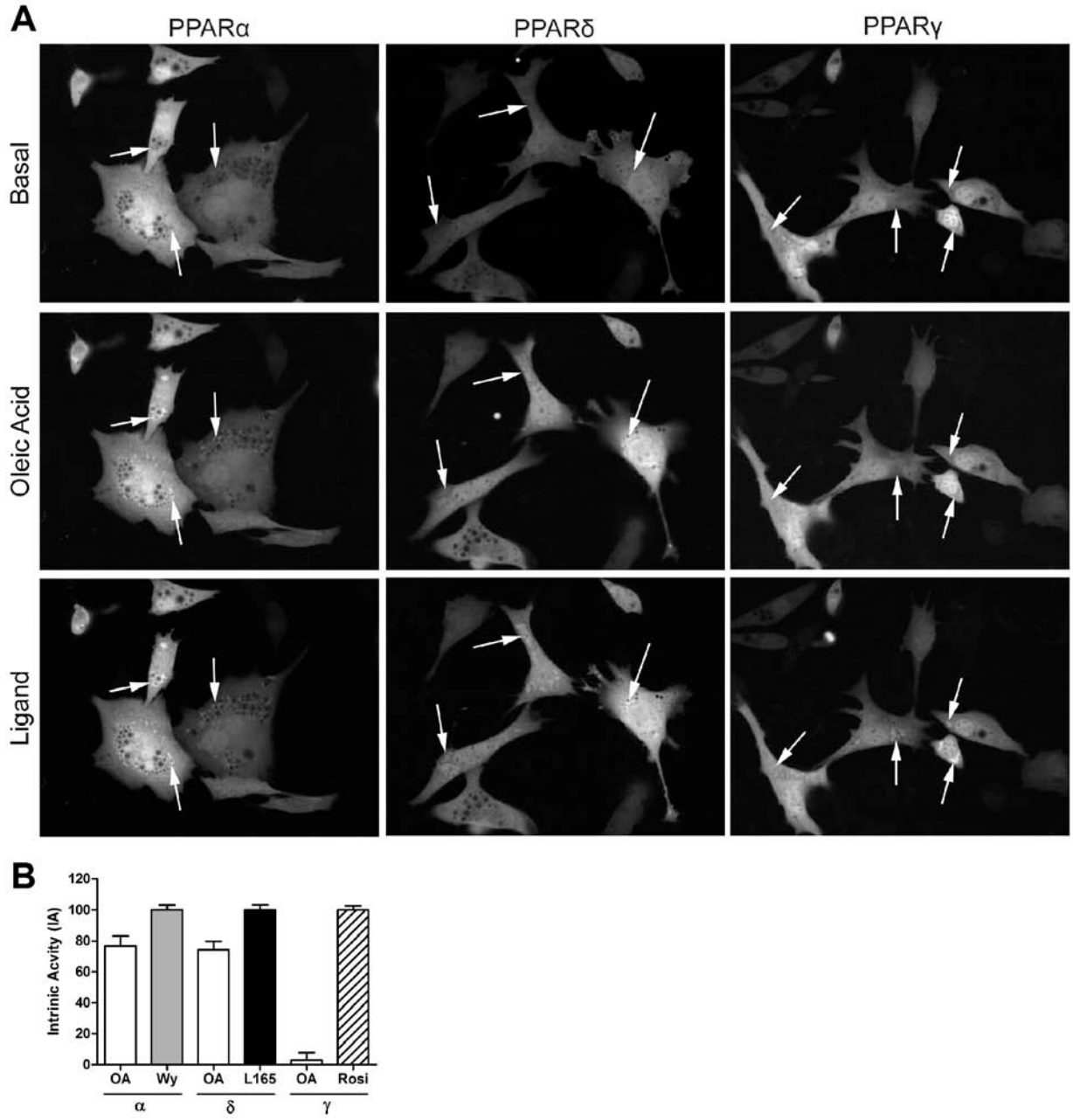

Figure 19. FAs are sufficient to activate PPARα and δ. Brown adipocytes transfected with reporters for PPARα, δ and γ were treated with 400 µM *OA* and the data was normalized to the Intrinsic Activity (IA) of the respective ligands Wy, L165 and Rosi.

We also tested the role of lipolytic products in activating PPARα and δ transcription using classical transcriptional reporter assays where the yeast Gal4 DNA binding domain is fused to the ligand binding domain of PPARs. As expected, β-AR stimulation induced transcription of Gal4/PPARα and Gal4/PPARδ reporters, and this effect was reduced with HSL inhibition (Figure 20). Overall, these results demonstrate that ligands for PPARα and δ are generated at the lipid droplet surface and increase the transcriptional activity of PPAR α and δ.

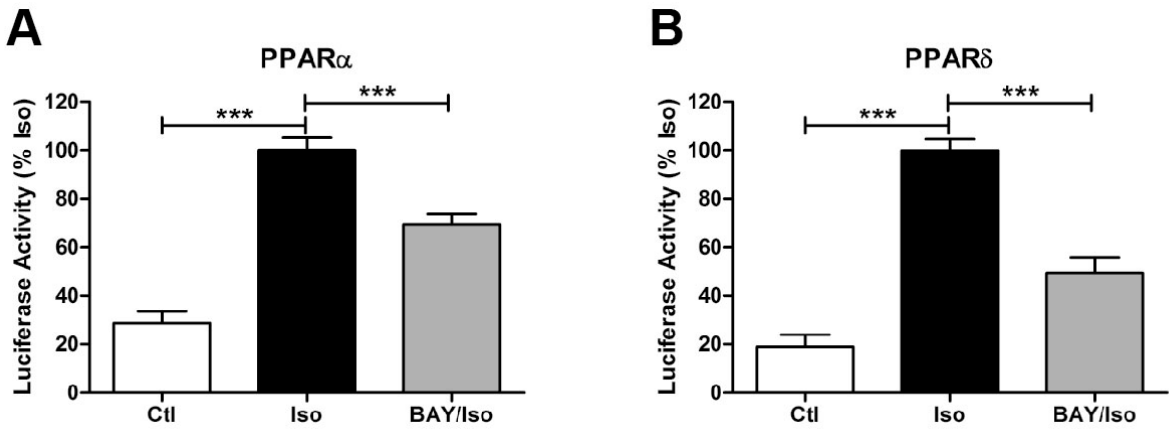

Figure 20. Lipolysis stimulates PPARα and δ transcriptional activity. Brown adipocytes transfected with β-galactosidase (β-gal), luciferase reporter and hPPARα-Gal4 or hPPARδ-Gal4 fusions were treated with vehicle or BAY (5 μM) and stimulated with *Iso* (10 μM) for 8 h. Luciferase reporter activity was normalized to β-gal activity and expressed as a percent of Iso, and statistical analysis was performed by one-way ANOVA to determine the effect of BAY or Iso.

5.5 Knockdown of ATGL reduces PKA upregulation of mitochondrial gene expression and fatty acid oxidation

The data thus far suggest that lipolytic products activate PPARα and δ to promote the expression of thermogenic genes. We wanted to test the functional significance of the relationship between lipase and oxidative metabolism, so to this end we examined the role of lipolysis in regulating mitochondrial gene expression and lipid oxidation. Activation of the β-AR

in BAs is known to increase mitochondrial biogenesis (Nechad, Nedergaard, and Cannon 1987), so we first tested whether direct activation of PPARα and δ was sufficient to promote mitochondrial gene expression. Although basal levels of long chain acyl-CoA dehydrogenase (LCAD) and medium chain acyl-CoA dehydrogenase (MCAD) mRNA were lower in shATGL BAs, direct agonists of PPARα and δ elevated their expression to similar levels observed in shCON cells (Figure 21A). Likewise, PPARα and δ agonists increased the expression of cytochrome C (Cycs) and cytochrome C oxidase IV (COXIV) to similar levels in control and ATGL shRNA expressing BAs (Figure 21A). These results demonstrate that activation of PPARα and δ is sufficient to elevate the expression of mitochondrial genes involved in FA oxidation and oxidative phosphorylation. We next tested whether stimulation with cAMP could increase the expression of mitochondrial genes equally in shCON and shATGL BAs. Basal gene expression of LCAD, MCAD, Cycs, COXII and COXIV were lower in shATGL cells. Stimulation with the cAMP analogue 8-Br-cAMP for 24 h induced the expression LCAD, MCAD, Cycs, COXII and COXIV in shCON BAs (Figure 21B). In contrast, the ability of 8-Br-cAMP to upregulate the expression of genes involved in lipid oxidation and oxidative phosphorylation was compromised in shATGL BAs (Figure 21B). Collectively, these results demonstrate that the defect in the ability of shATGL to upregulate the expression of genes involved in fat oxidation and mitochondrial function is at the level of ligand production.

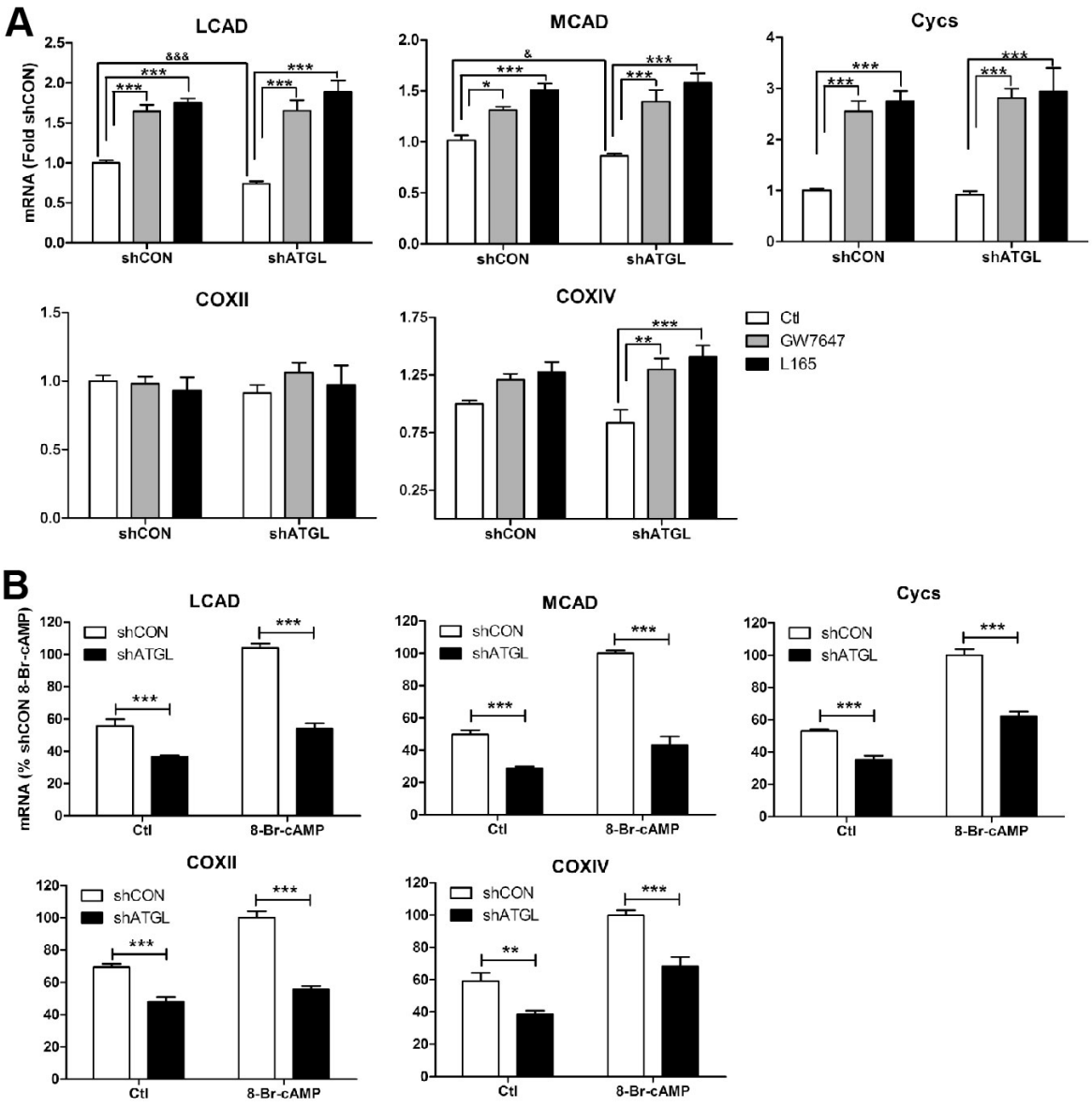

Figure 21. ATGL is required to maximally increase mitochondrial gene expression in response to cAMP stimulation in brown adipocytes. *A*, brown adipocytes expressing a control (*shCON*) or ATGL (*shATGL*) shRNA were treated with agonists against PPARα (GW7647; 1 μM) and δ (L165; 5 μM) for 24 h. mRNA levels were measured by QPCR, normalized to %PPIA and expressed as a fold of shCON. Measurements are from an average of three independent experiments performed in duplicate. The effect of GW7647 or L165 in comparison to Ctl is shown (***P < 0.001; **P < 0.01). The effect of ATGL knockdown is shown ($^{\&\&\&}$P < 0.001; $^{\&}$P < 0.05). *B*, Indicated cells were treated with H_2O (*Ctl*) or 8-Br-cAMP for 24 h and mRNA levels were quantified by QPCR, normalized to %PPIA and expressed as a percent of shCON 8-Br-cAMP. Measurements are from an average of four independent experiments performed in triplicate. The effect of ATGL knockdown is shown (shATGL) (***P < 0.001; **P < 0.01). Statistical analysis was performed by two-way ANOVA.

Finally, the effect of ATGL knockdown on mitochondrial biogenesis and mitochondrial activity was examined. There was no difference in mitochondrial DNA content between shCON and shATGL BAs (Figure 22A). Treatment of BAs with 8-Br-cAMP for 48h increased mitochondrial DNA content in shCON BAs and this effect was reduced in BAs with ATGL knockdown (Figure 22A). Next, we measured mitochondrial activity in permeabilized BAs in response to the addition of either a glycolytic (pyruvate) or FA substrate (palymitoyl-carnitine). 8-Br-cAMP increased coupled (ADP), and fully uncoupled (FCCP) respiration equally in shCON, and shATGL cells when pyruvate was used as the substrate for oxidation (Figure 22B). 8-Br-cAMP also increased oxidation of the FA substrate palmitoyl-carnitine (Pm-Carnitine) in shCON cells. However, in contrast to pyruvate, oxidation of Pm-Carnitine was reduced in cells with knockdown of ATGL. This difference between shCON and shATGL was more apparent when mitochondria were fully uncoupled (FCCP), and electrons were allowed to fully pass through the electron transport system without restriction (Figure 22B). These results indicate that FA oxidation is limiting for mitochondrial electron transport in the shATGL BAs.

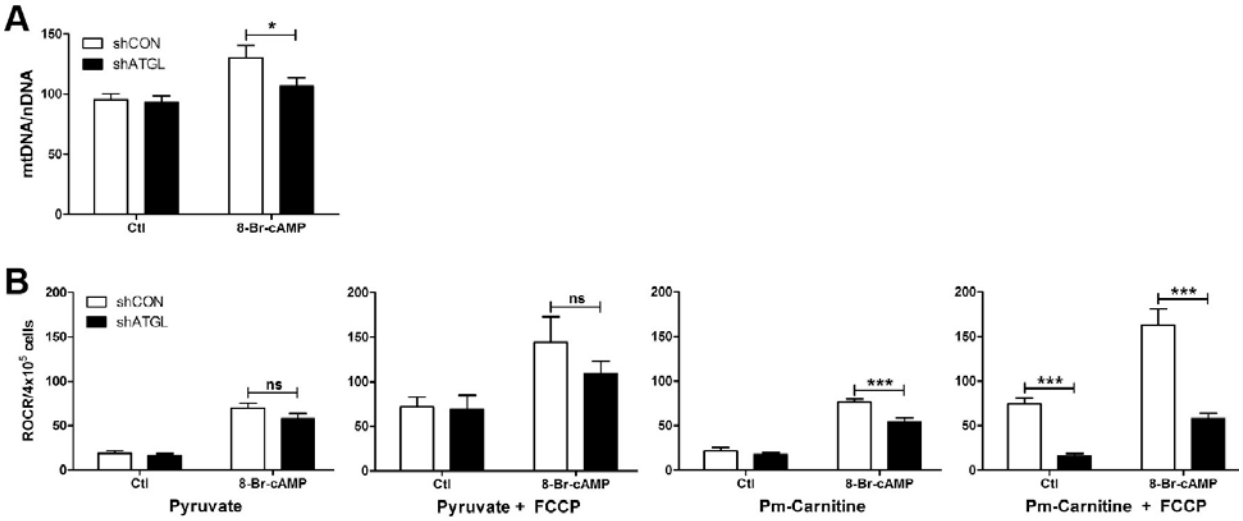

Figure 22. ATGL is required to maximally increase fatty acid oxidation in response to cAMP stimulation in brown adipocytes. *A*, mitochondrial DNA content (mtDNA) normalized to nuclear DNA (nDNA) in shCON and shATGL brown adipocytes in control state (*Ctl*) or treated with 8-Br-cAMP for 48 h. *B*, relative mitochondrial oxygen consumption rate (ROCR) in permeabilized shCON and shATGL brown adipocytes using pyruvate or palmitoyl-carnitine (Pm-Carnitine) as substrate. ADP driven (Pyruvate; Pm-Carnitine) and fully stimulated (Pyruvate + FCCP; Pm-Carnitine + FCCP) respiration is shown. Measurements are from an average of three independent experiments performed in duplicate. Statistical analysis was performed by two-way ANOVA to determine the effect of ATGL knockdown (shATGL) (***P < 0.001; *P < 0.05; ns, non-significant).

5.6 Chapter 5 Discussion

Activation of the β3-AR promotes thermogenesis in WAT by mobilizing FAs that both activate UCP1 and provide fuel for thermogenesis. β3-AR also increased the transcription of thermogenic genes that expand the capacity for oxidative metabolism. The pathways of lipolysis and gene transcription in BAT have previously thought to be independent events. This chapter explored the interactions between these pathways and demonstrates that FFAs potentiate PKA-dependent gene transcription in BAs.

We found that in BAs the optimal induction of PGC1α, PPARα, PDK4, and UCP1 mRNA by PKA activation requires lipolysis. That HSL inhibition had an additive effect in ATGL knockdown cells on the induction of thermogenic genes, indicating that lipolysis is a major pathway by which β -ARs regulate thermogenic gene expression BAT. Importantly, the lipolysis-dependent regulation of gene transcription was independent of the lipase, suggesting that both ATGL and HSL produce similar ligands for PPARs. The promoter elements of PGC1α (Barberá et al. 2001), PDK4 (Degenhardt et al. 2007), and UCP1 (Kozak et al. 1994) contain PPAR response elements (PPREs), which likely define the means by which lipolysis regulates their expression. We note that other pathways downstream of cAMP/PKA, like CREB (Karamitri et al. 2009), are important in synergizing with PPARs for maximal gene expression in BAT (Kozak 2011).

Of debate is the nature and source of endogenous ligands for PPARs. Our current results demonstrate PPARα and δ ligands are detected at the lipid droplet surface within minutes of PKA activation and can transcriptionally activate PPARα and δ over hours. The generation of ligands for PPARα/δ was rapid, profoundly suppressed by HSL inhibition and mimicked by exogenous OA. This indicates that mobilized FAs are likely endogenous ligands.

The identity of the ligand produced during lipolysis is likely a long chain non-esterified fatty acid, since increasing endogenous fatty acid levels by inhibition of ACSLs and CPT1 was sufficient to elevate gene expression. It is likely that multiple endogenous PPAR α and δ ligands exist, which vary by tissue, enzyme and metabolic status (Chakravarthy et al. 2009, Duncan et al. 2010, Ravnskjaer et al. 2010, Ruby et al. 2010, Sanderson et al. 2009, Sapiro et al. 2009).

Knockdown of ATGL significantly reduced induction of the rate limiting enzymes for mitochondrial fatty acid oxidation and impaired oxidation of fatty acids. Cells lacking ATGL were fully responsive to exogenous PPARα and δ ligands suggesting that there is no defect at the level of PPARs. Rather, ATGL is required for the full response to 8-Br-cAMP, indicating that the defect in gene regulation is due to the lack of ATGL-dependent PPAR ligands. The relationship between lipases and enzymes for β-oxidation suggests a feedback loop which permits FA oxidation to be closely matched to mobilization. Thus, lipolytic products activate PPARα and δ to enhance the oxidative phenotype of BAs (Figure 23).

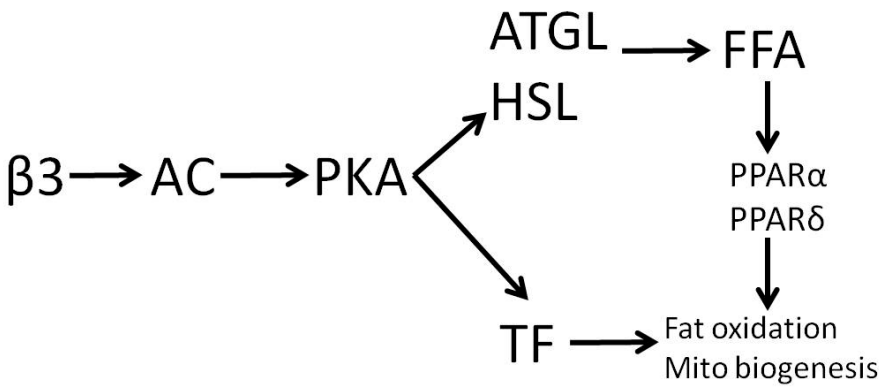

Figure 23. Lipolytic products activate PPARα and δ to promote lipid oxidation in BAT. β3-AR activation stimulates AC and PKA and subsequently ATGL and HSL to mobilize free fatty acids (FFA) Lipolytic products are detected by PPARα and δ to match lipid oxidation with the supply of FFAs. Other transcription factors (TF) such are CREB, likely function with PPARs for optimal PKA-mediated gene transcription.

Chapter 6. General Discussion

In the current study we have investigated the role of FA fate in promoting inflammation and oxidation in white and brown adipose tissue, which at first glance seem to be of opposing function. FAs have multiple fates (Figure 1), and an integrative understanding of how FAs signal within AT is lacking. Intracellular FAs produce inflammation and limit oxidative gene expression in WAT (Chapter 3 and 4). In contrast, lipolytic products in BAT activate PPARα and δ to promote an oxidative phenotype (Chapter 5). Excess FFAs can be toxic, and the mechanisms by which WAT and BAT deal with FA flux differ; however the outcome of limiting their detrimental effects is similar. The work presented in chapter 3 and 4 suggests that FFAs are toxic when their production exceeds efflux in WAT, which leads to the accumulation of intracellular FFA (Figure 24).

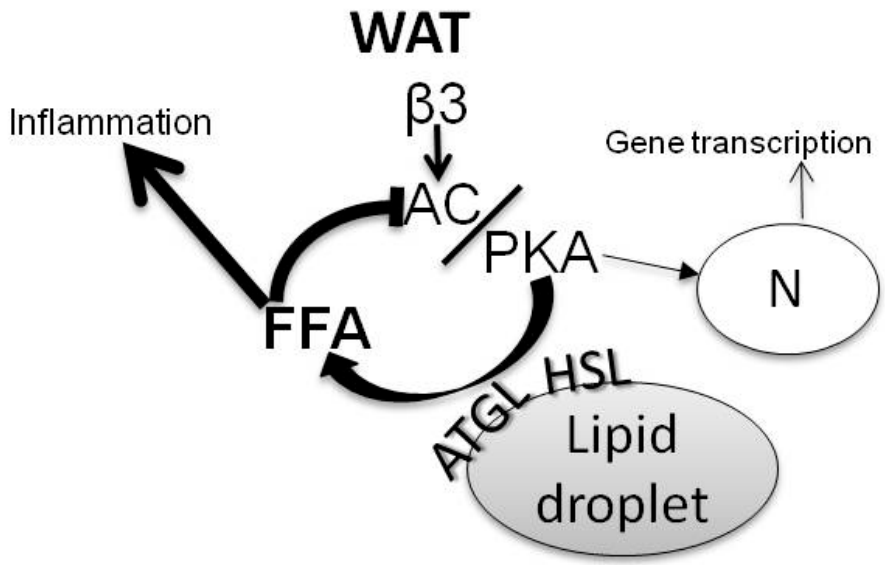

Figure 24. Lipolytic products in white fat balance production with efflux. FFA fate in WAT limits efflux by feedback inhibition on AC and by producing an inflammatory response. As a consequence, PKA-mediated gene expression is reduced.

In WAT, activation of lipolysis mobilizes energy for peripheral tissues. However, the excessive mobilization of FAs is toxic when production exceeds FA handling and/or oxidation. FA toxicity induced by β3-AR activation is initially limited by feedback inhibition on AC, which effectively diminishes lipolysis. Thus, feedback inhibition balances FA production with subsequent metabolism (Chapter 4). We found that FFAs limit cAMP production and reduce the expression of PKA-targeted genes by β3-AR activation. In this regard, treatments that limit intracellular FAs by increasing FA handling or oxidation (Seufert et al. 2004) would be expected to improve coupling of the β3-AR with PKA-targeted gene expression. Additionally, limiting intracellular FFAs in WAT would have additional anti-inflammatory and possibly anti-diabetic effects (Bensinger and Tontonoz 2008).

Activation of the β3-AR produces inflammation in WAT, a process marked by the expression of inflammatory cytokines, and subsequent recruitment of myeloid cells that surround fat cells (Granneman et al. 2005). The inflammation mounted by β3-AR activation is likely another means of limiting FAs, and is reminiscent of the inflammatory state associated with obesity. In obesity, activated macrophages surround necrotic adipocytes in order to offload lipid (Cinti et al. 2005, Murano et al. 2008). The inflammation induced by β3-AR activation can be considered a homeostatic response to lipid overload, and immune cells that are recruited to AT likely function to scavenge lipid and impart anti-lipolytic effects that further shut down lipolysis (Kosteli et al. 2010). However, β3-AR stimulation over days remodels WAT in a reparative process whereby dysfunctional fat cells are replaced by new cells derived from a population of PDGFRα+ progenitor cells (Lee et al. 2012). Under conditions of chronic β3-AR agonism these PDGFRα+ progenitor cells differentiate into BAs (inducible BA; iBA), whereby

EWAT takes on oxidative phenotype that diverts lipid towards β-oxidation. Interestingly, β3-AR activation does not produce inflammation in BAT, in which FFAs are coupled to oxidation (see below).

Although FA mobilization would be expected to initially have anti-diabetic properties, chronic β3-AR activation improves insulin sensitivity. At first, activation of the β3-AR suppresses insulin action in white adipocytes (Jost et al. 2005), as it elevates FFA metabolites. It may be that the acute availability of lipid as source of energy would initially diminish glucose utilization, a hypothesis first suggested by Randle and colleagues in the 60's (Randle et al. 1963). The improvement in insulin sensitivity seen with β3-AR agonists occur prior to changes in body weight (Cawthorne et al. 1992), and are associated with elevated FA oxidation in WAT, and a reduction in plasma FFAs (de Souza, Hirshman, and Horton 1997). Enhancement of FA oxidation in WAT requires PPARα, which also promotes resolution of inflammation mounted by β3-AR activation (Li et al. 2005). Interestingly, PPARα is highly expressed in BAT (Ahmadian et al. 2011), where lipolytic products are ligands for PPARs (see below).

In BAT, activation of β3-AR does not produce inflammation; rather mobilized FAs activate UCP1 to stimulate high rates of oxidation. We found that lipolytic products activate PPARα and δ to promote the expression of thermogenic genes. Lipase action was required to maintain FA oxidation in BAs. Thus, activation of PPARα and δ by ATGL and HSL is a homeostatic response to FFA mobilization that matches FA supply more closely with lipid oxidation (Figure 25).

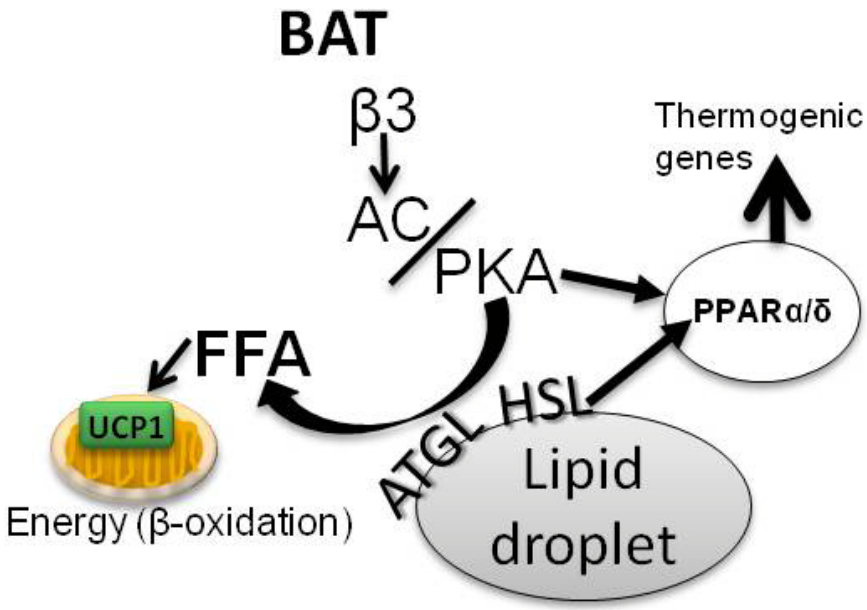

Figure 25. Lipolytic products in BAT match supply with oxidation. FFAs in BAT activate UCP1 and drives fatty acid oxidation. Lipolytic products in BAT are sensed by PPARα/δ to further upregulate lipid oxidation.

We observed contrasting effects with HSL inhibition in WAT vs. BAT with respect to the induction of oxidative genes. Furthermore, increasing intracellular FFAs in BAs enhanced the expression of oxidative genes, while in white adipocytes it reduced their induction. Intracellular FAs in WAT limit cAMP production, but whether similar mechanism exist in BAT seems unlikely as the induction of NOR-1, a known PKA-target, was not potentiated in BAT, or in cultured BAs. It is currently not known how FFAs might limit cAMP production in WAT, but not BAT. AC isoforms are differentially affected by nucleoside inhibitors and forskolin (Hurley 1999), which raises the possibility of differences in the sensitivity to FA antagonism. Interestingly, AC3 is highly expressed in BAT and is increased in response to neural and adrenergic stimulation, and corresponds with enhanced AC activity (Chaudhry et al. 1996, Granneman 1995). It may be that the differential expression pattern of AC isoforms between WAT and BAT accounts for the

differences in FFAs limiting oxidative gene expression. Furthermore, the contrasting effects of FFAs on gene expression in WAT and BAT could also be explained by the high levels of PPARα in BAT (Ahmadian et al. 2011).

We found that lipid droplets are a source of PPARα and δ ligands in BAs. However, FA spillover from adipose tissue leads to the accumulation of ectopic lipid in non-adipose tissues that reduces insulin sensitivity. The formation of lipid mediators such as DAGs and ceramides are critical in producing insulin resistance in skeletal muscle, liver and pancreatic beta cells (Glass and Olefsky 2012). It seems likely that the cellular source of FAs (*i.e.* lipid droplet vs. circulation) has an important role in determining their subsequent fate. For example, incomplete FA oxidation in muscle is thought to produce lipid mediators that cause insulin resistance (Koves et al. 2008). Furthermore, in the heart, the formation of endogenous PPARα ligands is thought to require hydrolysis from lipid droplets, as lipids from fatty acid uptake or lipoprotein lipase are not sufficient to serve as active signaling molecules (Haemmerle et al. 2011). How FAs traffic to the nucleus and activate PPARs is currently not known, but might involve FA binding proteins (Furuhashi and Hotamisligil 2008), or the recent identification of nuclear lipid droplets (Layerenza et al. 2013). Thus, an important area of future work would be to further understand cellular trafficking of FAs and the role that various organelles (*i.e.* mitochondrial, endoplasmic reticulum) play in their signaling.

Our data from brown fat suggests that stimulating lipolysis would be beneficial in activating BAT and likely enhancing its function. This finding has important clinical implications considering the identification of functional BAT in humans (Ravussin and Kozak 2009). Activation of lipolysis is of benefit in rodents, as overexpression of ATGL promotes an oxidative

phenotype in WAT (Ahmadian et al. 2009), and mice with genetic alterations that increase lipolysis have greater energy expenditure and are lean (Martinez-Botas et al. 2000, Nishino et al. 2008, Tansey et al. 2001). However, whether stimulation of lipolysis would have similar benefit in human is not known. Alternatively, human BAT could be activated with a dual PPARα/δ agonist. That ATGL and HSL are known PPAR targets (Rakhshandehroo et al. 2007) suggests that PPARα/δ agonism would create a feed forward loop between PPARs and lipases to further enhance BAT function. Direct agonist treatment would not require lipase activity as PPARα ligands rescue mice with ATGL deficiency in the heart from impaired cardiac function, and premature death (Haemmerle et al. 2011).

In summary, data generated here demonstrate the controlling FA fate could be a means of modulating AT function. Limiting the formation of intracellular FAs and ceramides and/or sphingolipids would be of benefit. Activation of PPARα and δ could promote lipid oxidation in BAT and likely other tissues, and limit lipotoxicity. Finally, the experiments proposed below should further expand the role of lipases and lipids in controlling inflammation and cellular metabolism.

Chapter 7. Future Directions

7.1 To further determine the signals which promote inflammation in WAT during β3-AR activation

Our early work indicated that the immune cells that are initially recruited to adipose tissue with β3-AR activation are likely neutrophils (Mottillo, Shen, and Granneman 2010). Further phenotyping of these cells by FACS will aid in understanding the interaction between immunity and metabolism in WAT. Previous work and that presented here, demonstrate that the expression of inflammatory cytokines and recruitment of immune cells is regulated by HSL (Mottillo, Shen, and Granneman 2007). Understanding whether lipid mediators generated by lipolysis (Finley et al. 2013, Spite et al. 2011), or the production of cytokines (Weisberg et al. 2006), are involved in immune cell recruitment of will be one area of focus. In addition, our data indicate that ceramides/sphingolipids are involved in the production of cytokines, therefore further interrogating ceramide and sphingolipid signalling in fat cells would be of interest (Hla and Dannenberg 2012).

7.2 To further determine the trafficking of fatty acids with in cells

Understanding the cellular trafficking of FFAs will be critical in determining whether other organelles can give rise to PPAR ligands. In addition, by expanding the use of the fluorescent PPAR sensors to other organelles this will provide a spatial and temporal understanding of how FAs traffic in cells. This will be part of larger goal to determine if specific pool FFAs give rise to PPAR ligands, or otherwise lead to the formation of bioactive lipids such as DAGs and ceramides.

7.3 To determine whether direct activation of lipolysis is of benefit in rodents

A larger goal of the laboratory is to identify small molecules that stimulate lipolysis at the level of lipases. Various genetic models indicate that enhancing lipolysis improves whole body metabolism (Ahmadian et al. 2009, Martinez-Botas et al. 2000, Nishino et al. 2008, Tansey et al. 2001). Future work will be focussed on determining if such small molecule activators of lipolysis promote fat loss and improve insulin sensitivity in mice. Moreover, determining the target tissues that are required to mediate the effects of such small molecules will be of interest.

7.4 To determine whether ATGL is required for the browning of white fat

Previous studies have demonstrated that deletion of ATGL converts BAT to a WAT-like tissue (Ahmadian et al. 2011). However, whether ATGL is required for the browning of WAT is not known. The appearance of BA in EWAT and inguinal WAT (IWAT) by β3-AR activation involve different mechanisms. BAs in IWAT appear from the direct conversion of pre-existing adipocytes (Barbatelli et al. 2010). In contrast, the appearances of BAs with chronic β3-AR agonism derive from a population of PDGFRα+ progenitors (Lee et al. 2012). By deleting ATGL specifically in AT in an inducible manner (via CreERT2 technology), we will be able to test whether adipocyte ATGL is required for the appearance of BA in WAT of mice. These studies could have important implication in understanding how BAs in adult humans are formed.

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ABSTRACT

FATTY ACID FATE IN DETERMINING OXIDATION AND INFLAMMATION IN ADIPOSE TISSUE

by

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Advisor: Dr. James G. Granneman

Major: Pathology

Degree: Doctor of Philosophy

Adipose tissue (AT) is a critical regulator of energy balance through its ability to store or oxidize free fatty acids (FFAs). White adipose tissue (WAT) functions as an anabolic organ to sequester and release FAs, in contrast brown adipose tissue (BAT) is a catabolic organ that oxidizes FAs. However, a comprehensive understanding of the role that FFAs play in the function of WAT and BAT is needed. Here we demonstrate that intracellular FAs enhance the expression of inflammatory cytokines by β3-AR activation in adipocytes, in which the expression of PAI-1 is partly mediated by the de novo synthesis of ceramides/sphingolipids. We also explored the relationship between lipolysis and oxidative gene expression in AT. β3-AR stimulation increased the expression of oxidative genes (PCG1α, UCP1 and NOR-1) in WAT of mice, which was greatly potentiated by inhibition of hormone sensitive lipase (HSL). In 3T3-L1 adipocytes, limiting lipolysis potentiated the induction of oxidative genes; while in contrast, promoting the accumulation of intracellular FAs suppressed their induction by β-AR stimulation. Interrogation of the β-adrenergic signalling pathway indicates that intracellular FAs inhibit adenylyl cyclase activity and thereby reduce PKA-mediated transcriptional activity. Partially

limiting lipolysis enhanced the induction of brown fat markers and mitochondrial electron transport chain activity in WAT, and facilitated fat loss in mice treated with a β3-AR agonist for five days. In contrast to the results observed in WAT, HSL activity was required for the induction of PGC1α, PPARα, PDK4 and UCP1 by β3-AR activation in BAT of mice. Similarly, lipolysis was required for the maximal induction of oxidative genes in cultured brown adipocytes (BAs), while increasing endogenous FAs elevated their expression. Pharmacological antagonism and siRNA knockdown indicate that PPARα and δ mediate the induction of oxidative genes by β-AR agonism. Dynamic imaging studies demonstrate that lipids droplets are a source of ligands that transcriptionally activate PPARα and δ. Finally, lipolysis was required to maintain the expression of mitochondrial genes, and FA oxidation in BA. Overall, results indicate that in WAT excess FFAs are toxic, and function to balance production with efflux, while in BAT FFAs promote oxidation, and match FA oxidation with supply. These finding suggest that limiting intracellular FAs is WAT, and activation of PPARα/δ would be of benefit in preventing the toxic effects of FAs, which could have implications for treating excess adiposity and diabetes.

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Education

B.Sc. Biological Sciences (honors) **1996-2001**
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Graduate Professional Scholarship	Wayne State University	2010-11

1. Department of Psychiatry and Behavioral Neurosciences 5th Annual Alumni Association Conference and Research Day. Basic Research Presentation Award 2010. $500. Wayne State University, April 30, 2010.
2. 14th Annual Graduate Student Research Day (GSRD). First Place, Oral Presentation Session One. $500. Wayne State University, September 23, 2010.
3. 15th Annual Graduate Student Research Day (GSRD). First Place, Oral Presentation Session One. $500. Wayne State University, September 29, 2011.
4. Deuel Conference on Lipids Travel Award. $400. March 6 – 9, 2012, Rancho Las Palmas, Palm Springs, CA

Published Papers (last six years)

1. **Mottillo E.P.**, X.J. Shen, J.G. Granneman (2007). Role of Hormone-Sensitive Lipase in Beta-adrenergic Remodeling of White Adipose Tissue. **Am J Physiol Endocrinol Metab**., 293(5), E1188-97.
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8. **Mottillo E.P.,** Block A.E., Leff T. and J.G. Granneman (2012). Lipolytic products activate peroxisome proliferator-activated receptor (PPAR) α and δ in brown adipocytes to match fatty acid oxidation with supply. ***J. Biol. Chem***. 287: 25038-48.

Oral Presentation

DEUEL Conference on Lipids. **Lipolysis fuels the nuclear receptor PPARs with ligands: a role for fatty acids in promoting oxidation in brown adipocytes**. March 6 – 9, 2012, Rancho Las Palmas, Palm Springs, CA.