Identification of TRPV4 as a Regulator of Adipose Oxidative Metabolism, Inflammation and Energy Homeostasis by a Chemical Biology Approach

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:10344925

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Identification of TRPV4 as a Regulator of Adipose Oxidative Metabolism, Inflammation and Energy Homeostasis by a Chemical Biology Approach

Abstract

PGC1α is a key transcriptional coregulator of mitochondrial biogenesis, oxidative metabolism and thermogenesis. We developed a quantitative high throughput screen to identify small molecules that can induce PGC1α expression in adipocytes. Small molecules antagonizing the TRPVs (Transient Receptor Potential Vanilloid), a family of ion channels, induced PGC1α expression in adipocytes. In particular, inhibition of TRPV4 increased expression of PGC1α, UCP1 and cellular respiration; conversely, chemical activation of TRPV4 repressed this pathway. Blocking TRPV4 in cultured adipocytes also reduced the expression of multiple proinflammatory genes that are involved in the development of insulin resistance. These effects of TRPV4 were mediated by the activation of ERK1/2. Finally, mice with a null mutation for TRPV4 showed higher energy expenditure with no change in movement or food intake, and were protected from diet-induced obesity, adipose inflammation and insulin resistance. This study links TRPV4 to robust pathways that offer therapeutic potential in obesity and related metabolic diseases.
# Table of Contents

**ABSTRACT** iii  
**TABLE OF CONTENTS** iv  
**LIST OF FIGURES** vii  
**LIST OF TABLES** ix  
**ACKNOWLEDGEMENTS** x

## CHAPTER 1

**Introduction**

- Obesity and Energy Balance 2  
- Brown Adipose Tissue and Uncoupled Respiration 5  
- Brown Adipose Tissue in Humans 7  
- Transcriptional Control of Brown Adipocyte Differentiation 8  
- PGC1α Co-activator 10  
- PRDM16 11  
- The Concept of Beige Fat 12  
- Obesity and Inflammation 13  
- Inflammation and Insulin Signaling 14  
- Stress-activated Kinases and NF-kB Signaling 14  
- The Initiation of Adipose Inflammation 16  
- Chemotactic Regulation 18  
- TRPV Family Channels and TRPV1 Pharmacology 20  
- TRPV4 and Other TRPV family Members 21  
- TRPs Function in Adipocyte Biology 23
CHAPTER 2

TRPV4 Regulates Oxidative Metabolism and Thermogenesis in Adipocytes

Introduction 26

Results 30

A Chemical Screen Identifies TRPVs as Negative Regulators of Pgc1a Expression 30

TRPV4 is Negative Regulator of Oxidative Metabolism, Thermogenic Pathway and Respiration in Adipocytes 34

TRPV4-deficient Mice Have Altered Expression of Thermogenic Genes in Adipose Tissue 38

Increased Energy Expenditure Protects TRPV4 Deficient Mice from Diet-induced Obesity 44

A Cell-autonomous Up-regulation of Thermogenic Program in Trpv4-/- Adipocytes 46

Discussion 51

The High Throughput Screen Platform for Mature Adipocytes 51

TRPVs and Adipocyte Biology 52

Materials and Methods 54

CHAPTER 3

TRPV4 Regulates Pro-inflammatory Pathways in Adipocytes

Introduction 59

Results 62

TRPV4 Positively Controls a Pro-inflammatory Gene Program 62

TRPV4 Deficiency Results in Reduced Pro-inflammatory Gene Expression in vivo 64

Trpv4/- Mice Have Reduced Inflammation in Adipose Tissue and Improved Glucose Tolerance 66

TRPV4 Deficiency Affects Adipocyte Pro-inflammatory Gene Program in a Cell-autonomous Manner 72
CHAPTER 4

Signal Transduction from TRPV4 to Transcription Regulation

Introduction 84

Results 87

TRPV4 Activation in Adipocytes Leads to Phosphorylation of ERK1/2 and JNK1/2 87

ERK1/2 Activation Primarily Mediates the Signal from TRPV4 Agonism to Gene Expression 89

Calcium Influx is Required for TRPV4 Agonism to Activate ERK1/2 89

Discussion 92

Materials and Methods 94

CHAPTER 5

Conclusion and Discussion

Conclusion 96

Connection between Adipose Thermogenesis and Inflammation 97

The Endogenous Activation of TRPV4 99

TRPV4 in other Tissues 101

References 103
List of Figures

Chapter 1

Figure 1. Energy balance at the organism level 3

Chapter 2

Figure 2-1. Summary of the high-throughput chemical screen 31
Figure 2-2. TRPV1 antagonists induce Pgc1α mRNA expression in adipocytes 33
Figure 2-3. Identification of TRPV4 as the major TRPV family member in adipocytes 35
Figure 2-4. Functional expression of TRPV4 in adipocyte and adipose tissue 37
Figure 2-5. TRPV4 negatively regulates oxidative metabolism and respiration in adipocytes 39
Figure 2-6. Trpv4-/- mice gain less body weight on high fat diet 41
Figure 2-7. Altered thermogenic programs in Trpv4-/- adipose tissue 43
Figure 2-8. Increased energy expenditure protects TRPV4 deficient mice from diet-induced obesity 45
Figure 2-9. Trpv4-/- mice have minimal change in thermogenic and oxidative pathways in interscapular brown fat and skeletal muscle 47
Figure 2-10. TRPV4 controls adipocyte thermogenic gene program in a cell-autonomous manner 49

Chapter 3

Figure 3-1. Loss of TRPV4 reduces pro-inflammatory gene expression in adipocytes 63
Figure 3-2. Activation of TRPV4 induces pro-inflammatory gene expression and protein secretion 65
Figure 3-3. Altered pro-inflammatory programs in Trpv4-/- adipose tissue 67
Figure 3-4. Trpv4-/- adipose tissue have less macrophage infiltration 69
Figure 3-5. Trpv4-/- mice have less inflammation and improved glucose tolerance 71
Figure 3-6. TRPV4 controls adipocyte pro-inflammatory gene program in a cell-autonomous manner 73

Figure 3-7. TRPV4 antagonist GSK205 represses pro-inflammatory gene expression and improves insulin resistance 76

Chapter 4

Figure 4-1. TRPV4 agonism leads to the activation of ERK1/2 and JNK1/2 88

Figure 4-2. ERK1/2 mediates the signal transduction from TRPV4 to gene expression 90
List of Tables

Table 1. Genes regulated by TRPV4-knockdown in 3T3-F442A adipocytes 60
Acknowledgements

I would like to thank my thesis advisor, Dr. Bruce Spiegelman. First of all, for his great guidance and inspiration to this work, his broad knowledge and critical thinking helped me carry the project through. I have been trained as an independent researcher, learning from how to identify an interesting and important question, design elegant experiments to scientific writing and presentation. Secondly, I am grateful that he provided the best research environment and resource as well as his efforts in communicating and collaborating with other leading groups in the field. Last but not least, I thank him for this wonderful role model: I have witnessed the passion, the vision and the perseverance of a true scientist in him. He is the best mentor a graduate student could ask for.

I would like to thank my thesis advisory committee, Drs. Gökhan S. Hotamisligil, Lewis Cantley, Chih-Hao Lee and Brad Lowell, for their valuable time, suggestions and encouragements. I would like to particularly thank Dr. Chih-Hao Lee again for his kind help and advice during my first rotation in his laboratory.

I am in debt to my collaborators and former/current colleagues for their supports. Drs. Patrick Seale and Rana Gupta, who taught me a lot in every aspect of in scientific research since I first came to the Spiegelman lab, helped me through my graduate study. Their knowledge, enthusiasm and brilliant mind made them wonderful persons to work with, to learn from and to be friends with. Thanks to
Drs. Sandra Kleiner, Jun Wu, Paul Cohen, Melin Khandekar, Alex Banks, Pontus Bostrom, Jorge Ruas, Jennifer Estall, Jang Hyun Choi, Yaniv Lustig, and Srikrupa Devarakonda for being great labmates. I also want to thank Rina Mepani, Dina Laznik, Diti Chatterjee Bhowmick and Yingying Zhang for their help.

I would like to thank Drs. Zoltan Arany and Bridget Wagner for their help with screen setup. Drs. Rajan Sah and Wolfgang Liedtke have been great collaborators and their help is sincerely appreciated.

Finally, I would like to thank my family, my wife Bingxin Lin and my parents, Shi Ye and Yunqin Ye, who always support and believe in me. Without their everlasting love and unconditional support, I would never come this far.
Chapter 1:

Introduction
Obesity and Energy Balance

Obesity is clearly an epidemic in the United States. According to the CDC, about one third of Americans are clinically obese (with a BMI more than 30) and another third are overweight (BMI>25). This is not only a problem in North America, but is also becoming a global health concern. The WHO estimated that 15% of the world population (1 billion) are overweight and more than 300 million are clinically obese. This trend in obesity is likely to contribute to the global public health burden, as obesity is a major risk factor for type 2 diabetes, cardiovascular disease, lipid disorders, and certain types of cancer (Haslam and James, 2005).

Fundamentally, obesity is a disorder of energy balance. In any system, the net energy intake must be balanced by the sum of total energy expenditure and energy storage. In mammals, energy intake comes from the absorption of digested food. Energy expenditure can be divided into three large categories (Figure 1.1). First is obligatory energy expenditure, which includes the energy need for basal cellular function, growth and reproduction. The second is used for physical activities, mainly via skeletal muscle. The third category is used for generating heat to maintain body temperature, a process called thermogenesis. Thermogenesis can be further divided into two parts: obligatory and adaptive. The former is the heat generated from all biochemical processes in the body, because essentially all chemical reactions result in energy loss in the form of heat. The latter refers to heat production in response to environmental challenges such as cold or diet.
Figure 1.1 Energy balance at the organism level. The net energy intake must equal to the sum of energy expenditure and energy storage.

Biological systems must follow the first law of thermodynamics. Therefore, obesity can only occur when energy intake exceeds energy expenditure, represented as an increase in energy storage, usually in the form of fat accumulation. Energy balance also provides the roadmap for treating obesity. One obvious avenue is to reduce energy intake, which can be achieved by either reducing the amount of food intake or reducing the absorption of nutrients by the digestive system.

There is likely a strong evolutionary force working against a strategy of restricting calorie intake, as during most of evolution, mammals faced a greater threat from starvation than from excess calories. For this reason, only a small portion of patients can achieve long-term weight loss, even with the combination of diet and exercise (Wing and Phelan, 2005). Even when medications targeting appetite have been effective in terms of weight control, because they affect neuronal
circuits involved in motivation and reward, they can also result in severe side effects. For example, a cannabinoid receptor 1 antagonist effectively reduced food intake and led to weight loss in obese patients, but was removed from the market due to an increased rate of suicide (Padwal and Majumdar, 2007). Other drugs have targeted lipid absorption in the intestine, but are associated with undesirable side effects and a lack of long-term efficacy (Melnikova and Wages, 2006). Bariatric surgery is probably the most effective among all the approaches in this category, but its invasive nature and cost make it less attractive as a treatment for what is becoming a societal problem.

Another way to alter energy balance is to increase energy expenditure. The portion used for basic cellular functions/growth, together with obligatory thermogenesis is difficult to alter by external intervention. Exercise, is a very popular approach for weight loss, and works by increasing energy output. Particularly when combined with diet, exercise can be very effective in altering the energy homeostasis and reducing body weight. However, patients have to follow a long-term exercise regimen to maintain the weight loss, which makes lasting success difficult (Wing and Phelan, 2005). Moreover, exercise is more useful for prevention or for patients in the early stages of obesity, as it can be challenging for morbidly obese patients or those with associated complications to exercise.

The last, but probably most interesting component in the equation is the energy used for adaptive thermogenesis. The significance and physiological role of adaptive thermogenesis in response to diet remains controversial (Rothwell et al.,
In contrast, cold-induced, particularly non-shivering thermogenesis has been well characterized at the cellular and organismal level in mammals. In response to cold, mammals need to quickly dissipate stored chemical energy, mainly from lipid, into large amounts of heat to keep the core temperature constant. In rodents and small animals, this process mainly takes place in the brown adipose tissue.

**Brown Adipose Tissue and Uncoupled Respiration**

White adipose tissue (WAT) is specialized for energy storage and usually has one large lipid droplet consisting of mainly triglyceride. White adipocytes have low mitochondrial content with limited oxidative capacity. In contrast, brown adipose tissue is specialized for dissipating chemical energy into heat. It has a large amount of mitochondria (the high cytochrome C content makes fat appear brown) and very high oxidative capacity. The most striking feature of brown adipose tissue is its capacity for uncoupled respiration in the mitochondria.

The mitochondrion is the primary organelle in cellular energy metabolism and typically accounts for more than 90% cellular oxygen consumption (Rolfe and Brown, 1997). In mitochondria from most cell types, the processes of breaking down chemical bonds and electron transport are highly coupled to the production of ATP, to provide fuel for essentially all the biochemical reactions in a cell. The mitochondria in brown fat are unique in that instead of producing ATP, they are specialized to generate heat from chemical reactions. Specifically, brown fat expresses a unique protein called uncoupling protein 1 (UCP1) in the
mitochondrial inner membrane. UCP1 allows the protons in the intermembrane 
space from the electron transport chain to re-enter the mitochondrial matrix 
without going through complex V to generate ATP. The leak itself releases the 
energy from the proton gradient into heat. More importantly, this futile cycle 
accelerates the influx through the electron transport chain and upstream 
reactions (such as TCA cycle and beta-oxidation), in which all intermediate steps 
produce heat.

It is clear that this uncoupling process is highly thermogenic, and also highly 
energy consuming by running fuel through futile cycles. In rats, for example, 
three grams of activated brown fat (1% of their body weight) can consume as 
much as 200% of the whole body basal metabolic rate (Foster and Frydman, 
1979). It is estimated that in humans, as little as 50 grams of activated brown fat 
is enough to consume up to 20% of the total metabolic rate (Rothwell and Stock, 
1983). Therefore, uncoupled respiration may provide a very powerful way to 
increase energy expenditure, and hence to alter the energy imbalance 
underlying obesity.

A nonspecific chemical uncoupler was actually used for treating obesity back in 
the 1920s. 2-4-dinitrophenol (DNP) was effective at increasing energy 
expenditure and reducing adiposity in humans (Harper et al., 2008). However, 
the excess heat production induced by this nonspecific uncoupler led to fatal 
fever in some patients and eventually caused the removal of this compound from 
clinical use. Nevertheless, the early example of DNP treatment demonstrated the 
efficacy of targeting uncoupled respiration as a therapeutic for obesity. It is
conceivable that, if one could specifically target uncoupling in adipose tissues rather than causing proton leak in every cell in the body, therapy targeting this pathway might be safe and effective.

**Brown Adipose Tissue in Humans**

In rodents, brown adipose tissue’s role in defending from cold challenge and obesity has been well established. In humans, brown adipose tissue has been identified in infants at the interscapular region and thought to be important for maintaining body temperature. However, after a few months of life, most likely due to the decrease in surface-to-volume ratio, the interscapular brown fat gradually disappears. For a long time, it was believed that adult humans do not possess functional brown fat and that adaptive thermogenesis is presumably mediated by alternative mechanisms in other tissues, such as skeletal muscle.

Several earlier reports suggested that “hot spots” were often identified around the neck and intercostal spaces near the spine of PET scans in patients (Hany et al., 2002; Tatsumi et al., 2004; Weber, 2004; Yeung et al., 2003). This observation triggered investigators to look more carefully for the possible presence of brown fat in adults. In 2009, three independent groups reported the “re-discovery” of brown fat in adult humans by using fludeoxyglucose (FDG) in combination with PET/CT technologies (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). It is now clear that adult humans do have supraclavicular UCP1-postive, brown-like adipose tissue that can be activated by cold exposure.
The physiological role of these depots in thermogenesis and metabolism is still not clear at this point.

**Transcriptional Control of Brown Adipocyte Differentiation**

Although the morphology, function and developmental origin of white and brown adipocytes are distinct, their cellular differentiation (adipogenesis) is similarly controlled by a largely overlapping transcriptional network. The molecular mechanism of white adipocyte differentiation has been extensively studied since the 1990s, after the identification of the important roles of PPAR-γ (Peroxisome proliferator-activated receptor-γ) and C/EBPs (CCAAT/enhancer-binding proteins) in controlling this process (Farmer, 2006). Briefly, PPAR-γ is the master regulator of adipogenesis, and is both sufficient and required for the formation of brown and white adipocytes. C/EBPs coordinate this transcription cascade, with PPAR-γ to promote and maintain adipose differentiation.

PPAR-γ is definitely necessary for brown fat differentiation (Rosen and MacDougald, 2006). However, when PPAR-γ was ectopically expressed in “naive” mesenchymal precursor cells, it drove the latter into white fat cells, suggesting there are additional cell type specific transcriptional components that specify the differentiation of brown adipocytes. This leads to the search for a brown fat specific co-activator of PPAR-γ. A brown fat-enriched co-activator of PPAR-γ, PGC1α (PPAR-γ coactivator-1α) was identified in 1998 using a yeast two hybrid system (Puigserver et al., 1998). Ectopic expression of PGC1α in mouse and human white adipocytes induced mitochondrial biogenesis and gave those cells
BAT-like thermogenic properties, such as increased expression of UCP1 and the deiodinase-2, with resultant higher cellular respiration (Puigserver et al., 1998; Tiraby et al., 2003). Although PGC1α whole body knockout mice develop severe hypothermia when they are challenged with cold exposure (Leone et al., 2005; Lin et al., 2004), the thermogenic program in cultured PGC1α-/- brown adipocytes appeared to be preserved by the closely related coactivator PGC1β (Uldry et al., 2006). Very recently, adipose-specific PGC1α knockout mice have been shown to have a defect in thermogenesis in adipose tissue and become more susceptible to metabolic disorders caused by a high fat diet (unpublished results).

Forkhead box C2 (FOXC2) is another transcription factor that is highly expressed in adipose tissue. Overexpression of FOXC2 in white fat in a transgenic mouse model strikingly increased the expression of UCP1 and PGC1α, causing a brown fat-like phenotypic switch in white adipocytes. In a transgenic mouse model, this phenotypic change rendered the animal resistant to diet induced obesity and metabolic disorders (Dahle et al., 2002). The lack of enrichment of FOXC2 in brown adipose tissue indicates that FOXC2 is probably not required for specifying the development of brown fat. It has been suggested that FOXC2 works by potentiating beta-adrenergic-cAMP-PKA signaling.

On the other hand, several factors have been shown to negatively regulate brown fat differentiation and/or function. The RB (retinoblastoma protein) family members: pRB (Hansen et al., 2004) and p107 (Scime et al., 2005), the nuclear receptor LXRα (Wang et al., 2008), the nuclear receptor co-repressor RIP140
(Christian et al., 2005), and many other transcription factors such as IKKe (Chiang et al., 2009), fsp27 (Toh et al., 2008), and twist-1 (Pan et al., 2009) have been shown to repress the expression of thermogenic genes particularly PGC1α and UCP1. Mice with deficiency in these genes have been reported to have a "browning" phenotype in white adipose tissue. However, the role of most of these genes in brown fat development remains to be further explored.

**PGC1α Co-activator**

PGC1α was originally identified as a coactivator of PPARγ in the control of the UCP1 promoter in brown fat cells (Puigserver et al., 1998). Subsequent work has illustrated that it binds to and coactivates most nuclear receptors as well as many transcription factors outside the nuclear receptor family (Handschin and Spiegelman, 2006). PGC1α plays a key role in mitochondrial biogenesis and oxidative metabolism in many tissues such as skeletal muscle and brown fat, linking mitochondrial biogenesis to the extracellular and extraorganismal environment.

PGC1α gene expression is induced in brown adipose tissue by cold exposure and by agents that activate the β-adrenergic system. Genetic ablation of PGC1α resulted in impaired cold-induced thermogenesis in vivo and a diminished cAMP-response *in vitro* (Lin et al., 2004; Uldry et al., 2006). Although PGC1α has a dominant role in thermogenesis, the loss of PGC1α did not affect the mass of brown adipose or the expression of many brown fat identity genes. Therefore, it is now believed that PGC1α functions as the central regulator of mitochondrial
biogenesis and thermogenesis in brown fat, but does not control its fate
determination. This led to a subsequent search for brown fat determination
factors that later identified PRDM16.

**PRDM16**

It has been noticed that precursors isolated from brown or white adipose tissue,
through an unknown mechanism, ‘remember’ their identity even after being
immortalized *in vitro* and cultured for many passages. For example, stromal-
vascular fraction cells from brown adipose tissue can be immortalized *in vitro* by
transducing SV40 into these cells. When these transduced BAT-derived
fibroblasts were induced to differentiate together with WAT derived fibroblasts
under the exact same conditions, the former maintained clear brown adipocyte
characteristics such as high thermogenic gene expression and robust cellular
uncoupled respiration. This intrinsic difference in precursor cells led to the
discovery of PRDM16 (PR-domain containing -16) as a brown fat determination
transcriptional regulator (Seale et al., 2007).

Ectopic expression of PRDM16 in mesenchymal fibroblasts or committed white
precursors induced a robust brown adipose program. Overexpression of
PRDM16 in a transgenic model potently induced brown characteristics in the
white adipose tissue and rendered the mice resistant against obesity and insulin
resistance (Seale et al., 2011).

Interestingly and strikingly, depleting PRDM16 in brown fat precursors not only
blocked brown fat adipogenesis, but drove these precursors to differentiate into
skeletal myocytes. Conversely, when PRDM16 was ectopically expressed in muscle precursors, it led to a robust brown fat differentiation in these cells. These results suggested that skeletal muscle and brown fat might have a very close developmental origin and that determination between these two cell fates might be controlled by PRDM16 (Seale et al., 2008).

To determine the development origin of brown adipose tissue, a lineage tracing experiment was performed in vivo using myf5, a gene that was believed to be exclusively expressed in muscle precursors. Surprisingly, it was discovered that interscapular brown fat originated from a myf-5 positive lineage that gave rise to skeletal muscle (Lepper and Fan, 2010; Seale et al., 2008). Importantly, this myf-5 positive lineage only gave rise to the “classical” interscapular brown fat depot, but not the emerging “brown fat-like cells” within white fat depots, in response to cold exposure or beta-agonist treatment.

The Concept of Beige Fat

Based on the lineage tracing with PRDM16, it is now known that there are two distinct kinds of brown fat cells. The classical type of brown fat is exemplified by the interscapular depot of rodents. These UCP1 expressing cells are derived from a muscle-like lineage that expresses Myf5/Pax7 during earlier development (Lepper and Fan, 2010; Seale et al., 2008). Pockets of UCP1-positive cells can also emerge in white fat depots under chronic exposure to cold or β-adrenergic stimulation (Cousin et al., 1992; Ghorbani and Himms-Hagen, 1997; Guerra et al., 1998; Himms-Hagen et al., 2000; Xue et al., 2005) as well as chronic treatment
with PPARγ agonists or prostaglandins (Petrovic et al., 2010). These cells do not come from a myf5-positive lineage (Seale et al., 2008) and have been called “beige”, “brite” (brown in white), “ectopic brown”, “systemic brown”, or “recruitable brown” fat cells (Ishibashi and Seale, 2010; Petrovic et al., 2010).

**Obesity and Inflammation**

Obesity has been known as a risk factor for insulin resistance and type 2 diabetes long before people understood the mechanistic connection between them. Over the past two decades, inflammation in adipose tissue associated with obesity has been appreciated as one of the most important factors linking increased adiposity and insulin resistance (Gregor and Hotamisligil, 2011; Horng and Hotamisligil, 2011). It was first discovered in 1993 that adipose tissue from obese mice expressed a higher level of TNF-α than adipose tissue from lean mice (Hotamisligil et al., 1993). Chronic, low-grade inflammation was identified to be present in adipose tissue from obese animals and humans (Hotamisligil et al., 1995; Hotamisligil et al., 1996). TNF-α is a secreted factor which potently inhibits insulin signaling. It acts on adipose tissues as well as other metabolic tissues such as muscle and liver to cause systemic insulin resistance. Besides TNF-α, many other pro-inflammatory cytokines were later found to be increased in obese adipose tissues, including Interleukin 6 (IL-6), interleukin 1- β (IL-1β) and monocyte chemotactic protein-1 (MCP1) (Donath and Shoelson, 2011). These contribute to the vicious cycle that amplifies and sustains the inflammation in adipose and other tissues.
Inflammation and Insulin Signaling

How inflammation affects insulin signaling has been intensively studied at the molecular level since the discovery of elevated TNF-α in obese tissues. Insulin receptors belong to the tyrosine kinase receptor family. In a simplified model, when insulin binds to its receptors, the receptors phosphorylate insulin receptor substrate proteins (IRS, 1-6) on tyrosine sites (Taniguchi et al., 2006; White, 2002). This insulin stimulated tyrosine phosphorylation on IRS is believed to be a crucial step for insulin signaling in all insulin sensitive cells. Exposing cells to inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, as well as free fatty acids can lead to serine phosphorylation on IRS proteins, which counteracts the tyrosine phosphorylation caused by insulin and therefore impaired insulin sensitivity.

This inhibitory serine phosphorylation is mediated by various stress-sensitive kinases that can be activated by pro-inflammatory cytokines and fatty acids, such as p38MAPK (Jiang et al., 2004), ERK1/2(Engelman et al., 2000), JNK1/2 (Gao et al., 2004; Suganami et al., 2005) and IKKβ (Gao et al., 2004). Among them, JNK1/2 and IKKβ have been most intensively studied for their functions in adipose inflammation and insulin resistance.

Stress-activated Kinases and NF-kB Signaling

JNK1/2 can be activated by TNF-α, ER stress, oxidative stress and free fatty acids (Aguirre et al., 2000; Ozcan et al., 2004; Wellen and Hotamisligil, 2005), all of which are known to be elevated in the obese state. Indeed, JNK1/2 was
reported to be activated in both dietary and genetic obese mouse models (Hirosumi et al., 2002). Activated JNK1/2 leads to phosphorylation on IRS1 and hence inhibits insulin signaling (Gao et al., 2004; Ozcan et al., 2004). It also activates transcription factors such as ELK1, ATF2 (activating transcription factor 2) and AP1 (Baud and Karin, 2001), which in turn further promote the expression of pro-inflammatory genes to amplify the vicious inflammation-insulin-resistance cycle. Mouse models with genetic JNK1 deficiency showed reduced adiposity, improved insulin sensitivity and became resistant to high fat diet induced insulin resistance (Hirosumi et al., 2002).

Both ERK1/2 and p38MAPK can be activated by interleukin-1 and TNF-α (Engelman et al., 2000; Hernandez et al., 2004; Jager et al., 2007; Lee et al., 2003). They both have been reported to be activated in obese animals (Bost et al., 2005; Jiang et al., 2004). The activated kinases also phosphorylate IRS1 and lead to decreased insulin sensitivity. ERK1-/- mice have been reported to have increased energy expenditure and are resistant to diet-induced obesity, although the effects on insulin resistance were confounded by differences in obesity and adipogenesis between the mutant and wild type mice (Bost et al., 2005). In a separate study, ERK1 deficiency partially rescued leptin-deficient (ob/ob) mice from insulin resistance by decreasing adipose inflammation (Jager et al., 2011).

IKKβ is another important kinase that has a role in inflammation and the development of insulin resistance. It can be activated downstream of cytokine receptors such as the TNF-α receptor (Gao et al., 2003b), as well as by toll-like receptors (TLRs) that bind to free fatty acids and pathogens (Arkan et al., 2005;
Cai et al., 2005). Besides directly phosphorylating IRS1 on serine sites, IKKβ also activates NF-κB signaling. The translocation of NF-kB from the cytoplasm to the nucleus ultimately leads to increased expression of many pro-inflammatory cytokines, including TNF-α. Heterozygous IKKβ knockout mice are partially protected from insulin resistance, at least in part due to its function in macrophages. Conversely, high-dose salicylates that inhibit IKKβ activity improved insulin sensitivity in animals and humans (Hundal et al., 2002; Yuan et al., 2001).

Overall, adipose inflammation and insulin resistance form a feed-forward cycle. In obesity, elevated secretion of pro-inflammatory cytokines, together with other metabolic stresses (many of which are caused by insulin resistance) leads to the activation of stress-sensing kinases such as JNK1/2, ERK1/2 and IKKβ. These kinases, on one hand, directly mediate the inhibitory serine phosphorylation on insulin receptor substrates (IRS) to further exacerbate insulin signaling. On the other hand, pro-inflammatory signals also converge at transcription factors such as AP1 and NF-κB, eventually leading to increased expression of those pro-inflammatory cytokines/adipokines which trigger this whole cascade in the first place. As obesity develops, this vicious cycle is continuously amplified and sustained, contributing to systemic insulin resistance and type 2 diabetes.

**The Initiation of Adipose Inflammation**

When TNF-α and inflammation was initially discovered in adipose tissue, it was believed that those inflammatory cytokines were secreted mainly from adipocytes
themselves. Later, it became clear that although adipocytes did secrete TNF-α and IL6, the majority of the cytokines from inflamed adipose tissue derived from the infiltrating immune cells (Weisberg et al., 2003; Xu et al., 2003). The presence of neutrophils, eosinophils and macrophages all have been reported in inflamed adipose tissue (Hotamisligil, 2006). While the role of other immune cells in adipose inflammation and insulin resistance is less clear, the importance of adipose tissue macrophages (ATM) has been well established over the last ten years. Macrophage infiltration is clearly associated with obese conditions and correlated with measures of insulin resistance, in both mice and humans. In severe obesity, these ATMs can make up to 40% of the cell population in adipose tissue and are a prominent source of pro-inflammatory cytokines such as TNF-α and IL6 (Weisberg et al., 2003; Xu et al., 2003).

Despite the significant role of macrophages in secreting cytokines and mediating insulin resistance, there are very few macrophages in adipose tissue in lean animals or humans. This raises the question of what signals first attract macrophages into adipose tissue in the obese state. Intuitively, some unknown changes must have taken place during the expansion of adipose tissue, likely in the adipocytes, which then send signals to the monocytes and/or other circulating immune cells to recruit them into the adipose tissue. The exact cause of this initial recruitment is largely unknown. Adipocyte cell death, mitochondrial dysfunction and increased ROS production, hypoxia, ER stress and nutrient overflow (fatty acid flux) have all been suggested to be the mechanisms by which early macrophage recruitment is initiated (Sun et al., 2011).
Chemotactic Regulation

Chemokines are small proteins that attract various immune cells, such as monocyte, neutrophils, T lymphocytes, basophils or eosinophils, from bone marrow or the circulation into tissues. They are characterized by their unique four highly-conserved cysteine residues: CXC chemokines which have two amino-terminal cysteine residues separated by one amino acid; and CC chemokines whose two amino-terminal cysteine residues are adjacent. As the chemokines present in inflamed adipose tissues can produced by adipocytes or immune cells, the origin of these molecules in the obese state is difficult to determine. However, because chemokines are necessary for the attraction of immune cells, it is reasonable to speculate that in the early phase of inflammation, the initial recruitment of macrophages must involve chemokines from non-immune cells, such as adipocytes, before there are significant amounts of infiltrating immune cells.

Many chemokines are associated with obesity and type 2 diabetes. In humans, increased MCP1 (CCL2), CXCL5 and CXCL8 have been shown to be associated with both obesity and diabetes. Others such as RANTES (CCL5), MCP3 (CCL7), MCP2 (CCL8), CCL11 and CCL13 have been reported to be elevated in obesity.

In mouse models, CCL2-5, CCL11, CXCL11, CXCL5, CXCL8 and CXCL10 have been found to be secreted from adipocytes, and their increased expression was associated with obesity and insulin resistance (Sell and Eckel, 2009).
Among them, MCP1 (CCL2) has been most extensively studied. *In vitro*, expression and secretion of MCP1 is regulated by insulin, TNF-α and IL6, consistent with its regulation *in vivo* in the context of obesity and diabetes (Fasshauer et al., 2004). A transgenic model with MCP1 overexpression in adipocytes mimics the phenotypes seen in obesity: increased macrophage infiltration in adipose tissue, insulin resistance and liver steatosis (Kamei et al., 2006). Conversely, studies using MCP1 deficient mice revealed that loss of MCP1 ameliorated adipose inflammation and insulin resistance in dietary or genetic obesity (Kanda et al., 2006). Consistent with the observations from the MCP1 knockout model (although there are conflicting results from other studies) (Inouye et al., 2007), one study using CCR2 (receptor for MCP1) knockout mice demonstrated that lack of CCR2 in high fat diet fed mice resulted in reduced macrophage infiltration, ameliorated inflammation and improved insulin sensitivity (Chen et al., 2005; Weisberg et al., 2006). However, the metabolic consequences of MCP1-CCR2 axis deficiency are relatively small, likely due to the redundancy between all the chemokines that attract monocytes.

Other than MCP1, other chemokines have not been thoroughly studied using genetic models. The characterization has been limited to their associations with insulin resistance or obesity.

**TRPV Family Channels and TRPV1 Pharmacology**

Transient Receptor Potential (TRP) cation channels are a superfamily of sensory channels wildly expressed across almost every tissue and cell type. Based on
their structure and function, they were further divided into seven subfamilies: TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPP (Polycystin), TRPML (Mucolipin), TRPA (Ankyrin) and TRPN (NOMP-C) (Nilius, 2007).

TRPV1 (Transient Receptor Potential Vanilloid receptor 1), also known as the capsaicin receptor, is the first cloned member of the TRPV family (Caterina et al., 1997). TRPV1 is a non-selective cation channel with calcium preference. Similar to other TRP family members, TRPV1 has a putative six-transmembrane domain and pore region located between the fifth and sixth transmembrane segments. As a nociceptor, TRPV1 can be activated by a range of physical and chemical stimuli, including capsaicin, heat (approximately 43°C), protons, bradykinin, nerve-growth factor, anandamide, prostaglandins, adenosine and ATP, polyamines and venoms from fish and insects (Szallasi et al., 2007).

TRPV1 became a popular target for pain control for several reasons. First, the agents or physical stimuli that activate TRPV1 are those cause pain sensation in humans or rodents (Knotkova et al., 2008; Szallasi and Blumberg, 1999). TRPV1 activity is indeed up-regulated in inflammatory conditions. Conversely, compounds that desensitize or antagonize TRPV1 alleviate pain-like behaviors or pain in rodents and human, respectively (Wong and Gavva, 2009). Moreover, TRPV1-/- mice showed attenuated thermal hypersensitivity after inflammation, indicating a role in mediating hyperalgesia (Caterina et al., 2000; Davis et al., 2000). In contrast to conventional approaches that either inhibits the production of inflammatory mediators or block the transmission of pain, targeting TRPV1
provides a novel and promising way to prevent pain by directly antagonizing sensor.

Because of this concept of manipulating TRPV1 for pain control, numerous efforts have been made to develop compounds that either directly antagonize this channel or desensitize it by using agonists. Particularly, several antagonists that have potent anti-hyperalgesic effects in animal models have been in clinical trials, including AMG517 (Amgen), AZD1386 (AstraZeneca), GRC6211 (Glenmark/lily), JTS-653 (Japan Tobacco) and MK2295 (Merck/Neurogen) (Wong and Gavva, 2009). A common side effect of TRPV1 antagonists, at least in animal models, is that essentially all of them caused a transient hyperthermia in vivo (Romanovsky et al., 2009). Interestingly, this side effect seems to be “on target” as the hyperthermia was not seen when those compounds were given to TRPV1 deficient mice (Romanovsky et al., 2009).

TRPV4 and Other TRPV family Members

The relatively mild phenotype seen in Trpv1-/- mice in terms of temperature and pain sensing (Caterina et al., 2000), at least in the non-stimulated state, appears to contradict its prominent roles in these pathways suggested by in vitro and pharmacological studies. This suggests that there are other closely related TRPVs in the family. Other TRPVs are likely to compensate for the loss of TRPV1 during development, indicating a redundancy in this system. On the other hand, the specificity of pharmacological approaches is also usually limited. The
combination of these two factors probably accounts for the discrepancy between the genetic and pharmacological observations.

There are six members in the TRPV family: TRPV1-6. While TRPV1-4 are thermosensitive, polymodal, calcium permeable but non-selective cation channels; TRPV5 and TRPV6 are highly selective for calcium and insensitive to temperature (Everaerts et al., 2010).

TRPV4 is closely related to TRPV1 with a 40.9% sequence identity. It was first identified as an osmosensor (Liedtke et al., 2000). Since then, many physical and chemical stimuli have been shown to activate TRPV4 (Nilius et al., 2004), including warmth (Guler et al., 2002; Watanabe et al., 2002), mechano-stimulation (Gao et al., 2003a), endocannabinoids (Watanabe et al., 2003) and bisandrographolide A (BAA) (Smith et al., 2006). TRPV4 is widely expressed in many tissues, including kidney, lung, fat, bladder, brain, skin, brain, dorsal root ganglia, liver, testis and heart. Also, its expression is not limited to excitable cells but it is also present in non-excitable cells, such as epithelial cells, chondrocytes, and osteoclasts (Everaerts et al., 2010).

Corresponding to its broad expression pattern, many functions of TRPV4 in various cells/tissues have been explored since its discovery. Among them, osmolarity sensing/regulation in CNS (Liedtke and Friedman, 2003), bone remodeling (Masuyama et al., 2008) and thermosensation (Lee et al., 2005) are the best characterized functions of TRPV4 based on a genetic TRPV4 deficiency model. In addition, other potential roles in epithelial cells, endothelial cells,
bladder urothelium and smooth muscle cells have been suggested in cellular models but the physiological importance of these roles required more in vivo studies (Everaerts et al., 2010).

Notably, there are three recent studies linking TRPV4 mutations to human diseases. In one study, two heterozygous amino acid substitution mutations on TRPV4 (C946CT and G806A) resulted in familial scapuloperoneal spinal muscle atrophy (SPSMA) and Charcot-Marie-Tooth disease type 2C (CMT2C) in two unrelated families (Deng et al., 2010). The other study identified an additional heterozygous C946T mutation also causing SPSMA or CMT2C (Auer-Grumbach et al., 2010). The third study showed another two CMT2C-causing heterozygous mutations (C805CT and G806GA) in TRPV4 (Landoure et al., 2010). Despite the striking phenotype, however, the nature of these mutations is still controversial; as there are conflicting results as to whether these are gain-of-function or loss-of-function mutations. Further study is needed to clarify the mechanism of these channelopathies.

**TRPs Function in Adipocyte Biology**

TRPs, particularly TRPV1, have been reported to function in adipocytes. Activation of TRPV1 by capsaicin has been reported to inhibit adipogenesis in 3T3-L1 adipocytes in vitro (Zhang et al., 2007). Administration of capsaicin in high fat fed mice was shown to reduce adiposity by inhibiting adipogenesis. However, inhibition of adipogenesis in vitro can be highly nonspecific and the approach of inhibiting adipose development is unlikely to change energy balance
in vivo. Most likely, the difference in obesity observed in the study was due to reduced food intake caused by capsaicin administration. The other report using Trpv1-/- mice reached the opposite conclusion from the first report. Genetic loss of TRPV1 resulted in higher energy expenditure and less weight gain (Motter and Ahern, 2008). Moreover, the authors failed to detect TRPV1 expression in adipocytes and therefore suggested the effect was not cell-autonomous in adipocytes. Overall, based on these two conflicting reports, it is difficult to establish whether TRPV1 has a positive or negative role in obesity.

TRPM8, another TRP channel that is activated by cold and menthol has recently been reported to control a thermogenic program in brown adipocytes. Treating high fat fed mice with menthol, a TRPM8 agonist, increased BAT-mediated thermogenesis and therefore reduced obesity and improved insulin resistance in these mice, suggesting a previously unrecognized role of TRPM8 in promoting BAT thermogenesis (Ma et al., 2012).
Chapter 2:

TRPV4 Regulates Oxidative Metabolism and Thermogenesis in Adipocytes
Introduction

Obesity is fundamentally a result of an imbalance in energy intake and energy expenditure. Manipulating the components of the energy equation is a straightforward approach that could ultimately alter the progression of obesity and development of obesity-related metabolic consequences. Brown adipose tissue is a specialized organ that dissipates stored chemical energy into heat, a process called thermogenesis that significantly contributes to whole body energy expenditure in animals. However, in obesity, most of the excess adipose tissue is white adipose tissue, which has very little capacity for utilizing stored fat for heat (thermogenic capacity). Therefore, converting white into brown fat, or imparting brown fat-like thermogenic properties on white adipose tissue is an appealing approach to alter the energy imbalance that causes obesity.

The functional conversion between white and brown adipocytes is known to happen under certain physiological conditions. For example, chronic cold exposure, beta-adrenergic agonism and TZD treatment, are all known to promote brown-fat like properties in white adipose tissue in animals models (Cousin et al., 1992; Ghorbani and Himms-Hagen, 1997; Guerra et al., 1998; Himms-Hagen et al., 2000; Xue et al., 2005). Transcriptional control has been shown to be important in this conversion. Several transcription factors/ co-factors, such as PGC1α, PDRM16, FOXO1 and CEBP/b have been shown to play important roles (Kajimura et al., 2010). Ectopic expression of these transcription factors by viral vectors or transgenic approaches in white adipose tissue or cultured adipocytes
can activate the oxidative and thermogenic gene programs, and therefore alter energy metabolism.

Among these factors, PGC1α appeared to be an appealing target. We hypothesized that increased PGC1α expression would likely alter the gene programs toward a more oxidative and thermogenic direction in white adipocytes, for the following reasons. First, the expression level of PGC1α was actively regulated under different physiological conditions. For example, cold exposure or cAMP stimulation significantly up-regulates PGC1α expression whereas high-fat diet and obesity effectively down-regulates it. Secondly, changes in PGC1α expression have been shown to have profound biological effects in the gene programs that are controlled by PGC1α in different systems (Lin et al., 2002; Puigserver et al., 1998; Tiraby et al., 2003). Lastly and importantly, as a co-activator rather than an individual transcription factor or enzyme, PGC1α controls a whole set of genes that are important in oxidative metabolism and thermogenesis, such as enzymes in beta-oxidation, electron transport chains, mitochondrial biogenesis, uncoupling protein and ROS clearance (Spiegelman and Heinrich, 2004). Hence, it is believed that an increase in PGC1α would result in a “coordinated” increase in cellular energy metabolism.

It is conceivable that increased expression of PGC1α would lead to increased mitochondrial content, elevated oxidative capacity, and promote thermogenesis in white adipocytes, which we here define as the “browning” of the white fat. However, viral or transgenic approaches are not a practical means of manipulating those pathways in humans for therapeutic purposes. While
chemical biology has been mainly considered as a tool for drug development in the pharmaceutical industry, recent advances in chemistry and high-throughput screening technologies have allowed academic laboratories to use this approach to look for small molecules that regulate important biological targets. It can be used to establish proof of concept of the “druggability” of targets and provide preliminary scaffolds which others can utilize for drug development.

A similar screen for chemical inducers of PGC1α has been done in myotubes. After realizing that elevated PGC1α in muscle plays an anti-dystrophic and anti-atrophic function, we previously screened for drugs and drug-like molecules that elevate PGC1α in primary murine muscle cells (Arany et al., 2008). Several inhibitors of microtubules and protein synthesis were identified as PGC1α inducers. This illustrated that screening for activators of PGC1α expression could identify compounds capable of increasing mitochondrial action. Conversely, when a screen for chemicals that could alter mitochondrial function was carried out, an overlapping set of regulators of PGC1α was uncovered (Wagner et al., 2008). Unfortunately, none of these compounds had an activity/toxicity ratio that was favorable for animal or human studies.

In this study, we have screened a chemical library for compounds that could increase PGC1α gene expression in adipocytes. We used a library of 3000 compounds, most of which are either FDA-approved drugs or have known biological targets. This selection is critical as we not only are looking for drug-like small molecules that can be used to demonstrate the “druggability” of PGC1α,
but may also identify novel connections between known signaling pathways and the molecular control of PGC1α expression.

We chose 3T3-F442A cell as our screening platform. 3T3-F442A is a clonal adipogenic cell line from immortalized Swiss 3T3 cell lines. They undergo spontaneous differentiation into adipocytes, and the process can be enhanced with insulin (Green and Kehinde, 1976). There are several reasons that 3T3-F442A cells were used for the screen. First, 3T3-F442A cells are clonal, immortalized cells, which could give highest reproducibility with little variation between experiments. Second, they can differentiate into adipocytes with minimal external hormonal stimulation (insulin alone), which minimizes any possible drug-drug interaction in the compound treatment step in the screening and also makes the screen setup simple. Third, 3T3-F442A is the only adipocyte cell line that can form fat pats when they are injected into mice, indicating they are a very close alternative to in vivo systems (Mandrup et al., 1997). Last but most importantly, it is known that transcription factors that are important for the “browning” in vivo; can function normally in 3T3-F442A adipocytes in terms of regulating thermogenic gene expression and cellular physiology. On the other hand, many of those factors failed to regulate those pathways in 3T3-L1 cells, another popular model system for adipocyte biology (unpublished observation).
Results

A Chemical Screen Identifies TRPVs as Negative Regulators of Pgc1α Expression

We performed a quantitative PCR-based chemical screen to identify small molecules that can induce Pgc1α mRNA expression in white adipocytes. Fully differentiated 3T3-F442A adipocytes were treated with a chemical library of 3,000 drugs and drug-like compounds for 20 hours; mRNA from treated cells was then harvested and analyzed by qPCR to quantify the expression of Pgc1α (Figure 2.1). 33 primary candidates were identified from the screen, many of which overlapped with the results from the myotube Pgc1α screen, including 9 protein synthesis inhibitors and 5 mitochondrial respiration chain inhibitors. Of note, the screen also identified several ion channel inhibitors, protein modification inhibitors, and lipid derivatives.

AM-251, a cannabinoid receptor 1 (CB1) antagonist was identified as one of the primary hits. It induced Pgc1α mRNA 10 fold at 20uM (Figure 2-2 A). AM-251 is a structural analogue of another well-known CB1 antagonist rimonabant (Lan et al., 1999), an anti-obesity drug that was in clinical use in Europe but later was withdrawn due to psychiatric side effects. Although AM-251 is annotated as a CB1 antagonist, two other CB1 antagonists, SLV319 (Lange et al., 2004) and CAY10508 (Muccioli et al., 2006), failed to induce Pgc1α at any dose tested (0.2-20uM) (Figure 2-2 A). Importantly, other molecular targets of AM251 or rimonabant have been reported when these compounds were used at 10uM or
Figure 2-1. Summary of the high-throughput chemical screen. The results were presented as dCT (the CT number difference between *Pgc1a* and *Tbp*) from each sample. Each point on X-axis represents one 384-well plate treated with corresponding library plate. In general, the lower the dC indicates the more *Pgc1a* mRNA was expressed in the cells from that well.
above including TRPV1 (De Petrocellis et al., 2001; Zygmunt et al., 1999). As shown in Figure 2-2 B, two TRPV1 antagonists, AMG9810 and BCTC, increased \( Pgc1a \) mRNA expression in 3T3-F442A adipocytes in a dose-dependent manner. Moreover, key transcriptional targets of PGC1\( \alpha \) such as Cytochrome C (CytC) and \( Ucp1 \), were also increased at the mRNA level both basally and after cAMP-stimulation (Figure 2-2 C).

AMG9810 is known to antagonize TRPV1 but can also antagonize closely related TRPVs, such as TRPV2, TRPV3 and TRPV4, at the micromolar doses used here (Gavva et al., 2005). We therefore compared the mRNA expression of \( Trpv1 \), \( Trpv2 \), \( Trpv3 \) and \( Trpv4 \) in 3T3-F442A adipocytes. As shown in Figure 2-3 A, mRNAs encoding \( Trpv1 \), \( Trpv2 \) and \( Trpv4 \) were expressed in 3T3-F442A adipocytes, with \( Trpv4 \) being expressed at the highest level. To determine which of these channels were regulating \( Pgc1a \) expression, we used shRNA-mediated knock-down of each of the expressed TRPVs with lentiviral expression vectors. As shown in Figure 2-3 B, \( Trpv1 \), \( Trpv2 \) and \( Trpv4 \) mRNA were each significantly reduced by the corresponding shRNA expressed from lentiviral vectors, with no apparent cross-regulation. None of the shRNAs appeared to affect adipose differentiation \textit{per se}, as indicated by the similar expression of the adipose-selective gene \( aP2 \) (Figure 2-3 C). \( Pgc1a \) mRNA was strongly induced by the shRNA against TRPV4; shRNA against TRPV1 also had a small effect (Figure 2-3 D). This functional data, along with the fact that the expression of \( Trpv4 \) mRNA was 10 times higher than that of \( Trpv1 \) in these cells, strongly suggested that
Figure 2-2. TRPV1 antagonists induce Pgc1α mRNA expression in adipocytes. QPCR analysis of Pgc1α mRNA in fully differentiated 3T3-F442A adipocytes after 24-hour treatment with indicated CB1 antagonists (A) or TRPV1 antagonists (B). All chemicals were used at three doses: 0.2, 2 and 20uM, except AM251 (20uM). (C) QPCR analysis of Pgc1α, CytC and Ucp1 mRNA in adipocytes treated with 20uM AMG9810 or DMSO, at basal level or after Forskolin (10uM) stimulation. Data are presented as mean ± sem. Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, compared to control group.
TRPV4 was the dominant TPRV family member regulating the induction of \textit{Pgc1a} mRNA by the chemical inhibitors.

\textbf{TRPV4 is a Negative Regulator of Oxidative Metabolism, Thermogenic Pathway and Respiration in Adipocytes}

TRPV4 is a calcium permeable, non-selective ion channel that was first identified as an osmolality sensor (Liedtke et al., 2000; Strotmann et al., 2000). Since then, many physical and chemical stimuli have been shown to activate TRPV4 (Nilius et al., 2004), including warmth (Guler et al., 2002; Watanabe et al., 2002), mechano-stimulation (Gao et al., 2003a), endocannabinoids (Watanabe et al., 2003) and bisandrophorolide A (BAA) (Smith et al., 2006). Adipose tissue was shown to have one of the highest levels of \textit{Trpv4} mRNA expression (Liedtke et al., 2000). We also found that in general \textit{Trpv4} expression was higher in white adipose tissues (including epididymal, inguinal and retroperitoneal fat) than in brown adipose tissue (Figure 2-4 A).

We used retroviral vectors expressing an shRNA against TRPV4 or GFP to make stable cells with altered TRPV4 expression for biochemical and bioenergetic analyses. Again, the ectopic retroviral shRNA did not appear to effect adipocyte differentiation \textit{per se} (Figure 2-4 D). We first examined if there were functional TRPV4 channels present in 3T3-F442A adipocytes. TRPV4 protein was detected at the predicted molecular weight, by western blot (Figure 2-4 B). In addition, we used intracellular calcium measurement as a functional assay to test for TRPV4 conductivity.
Figure 2-3. Identification of TRPV4 as the major TRPV family member in adipocytes. (A) Normalized mRNA expression of \(\text{Trpv1}, \text{Trpv2}, \text{Trpv3}\) and \(\text{Trpv4}\) in 3T3-F442A adipocytes, by QPCR. (B) \(\text{Trpv1}, \text{Trpv2}\) and \(\text{Trpv4}\) mRNA levels in adipocytes infected with scrambled (SCR), shTRPV1, shTRPV2 or shTRPV4 lentivirus. \(aP2\) (C) and \(\text{Pgc1a}\) (D) mRNA levels in these adipocytes. Data are presented as mean ± sem. Student’s t-test was used for single comparisons. * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\), compared to control group.
GSK1016790A, a potent and selective TRPV4 agonist (Thorneloe et al., 2008; Willette et al., 2008), induced a robust and rapid increase in intracellular calcium in adipocytes at 100nM. This calcium increase by GSK1016790A treatment was highly dependent on the presence of TRPV4, as it was largely abolished by the shRNA against TRPV4 (Figure 2-4 C).

Pgc1a mRNA expression was 3-10 times higher in adipocytes expressing shRNA against TRPV4 with this retroviral system, compared to controls (Figure 2-5 A). At the basal state, TRPV4 knockdown adipocytes did not have significant higher UCP1 mRNA expression. β-adrenergic signaling is important for the induction of PGC1α and its target genes in thermogenesis. When cells were exposed to norepinephrine, mRNA expression of Pgc1a and its thermogenic target Ucp1 was robustly increased (4-7 fold) in the TRPV4 knock-down cells compared to controls (Figure 2-5 A). PGC1α is known to drive the expression of many genes involved in mitochondrial oxidative phosphorylation, including cytochrome c (CytC), and the cytochrome C oxidative (COX) subunits (CoxIII, Cox4ii, Cox5b, Cox7a and Cox8b) which are important for the electron transport chain on the mitochondrial inner membrane. We observed higher mRNA expression of these genes (1.5-2fold) in TRPV4-knockdown adipocytes compared to controls (Figure 2-5 B). In addition, the TRPV4-knockdown adipocytes showed significantly higher expression of proteins present in all five OXPHOS complexes (Figure 2-5 C).
Figure 2-4. Functional expression of TRPV4 in adipocyte and adipose tissue. (A) QPCR analysis of TRPV4 mRNA in interscapular brown fat (BAT), inguinal (ING), axillary (AXL), epididymal (EPI) and retroperitoneal (RP) fat. (B) TRPV4 protein in 3T3-F442A adipocytes were infected with retrovirus expressing shTRPV4 or shGFP. (C) Oil-Red-O staining (red) for lipid accumulation. mRNA levels of general adipocyte markers (aP2, Adiponectin and PPARγ) were also determined. (D) Intracellular calcium measurement, the calcium level was presented as ratio of 340nm/380nm emission from Furo-2.
The increased expression of *Pgc1α*, *Ucp1* and other mitochondrial genes suggested that TRPV4 inhibition caused white adipocytes to develop brown fat-like characteristics, which we termed “browning” here. To determine the impact of this browning gene program on cellular physiology, oxygen consumption was measured in adipocytes in a closed chamber with an oxygen sensitive Clark electrode at the bottom. As shown in Figure 2-5 D, TRPV4 knockdown has significant effects on the basal, uncoupled and maximal cellular respiration rate. Adipocytes with reduced TRPV4 showed a 40% increase in basal respiration, a 30% increase in uncoupled and a 30% increase in FCCP-stimulated maximal respiration, relative to controls, indicating the elevated mitochondrial oxidative gene program was associated with increased cellular respiration in these cells.

We next examined whether chemical activation of TRPV4 would have the opposite impact on the same pathways. The TRPV4 agonist GSK1016790A was added to mature 3T3-F442A adipocytes for 48 hours. While there was no difference in adipocyte differentiation, as assessed by *aP2* gene expression, GSK1016790A repressed the expression of mRNAs encoding *Pgc1α*, *Ucp1* and *Cox8b* in a dose-dependent manner (Figure 2-5 E). Taken together, these data strongly suggest that TRPV4 functions as a negative regulator of PGC1α and oxidative metabolism in white adipocytes.

**TRPV4-deficient Mice Have Altered Expression of Thermogenic Genes in Adipose Tissue**
Figure 2-5. TRPV4 negatively regulates oxidative metabolism and respiration in adipocytes. 3T3-F442A adipocytes were infected with retrovirus expressing shTRPV4 or shGFP. (A) *Pgc1α* and *Ucp1* mRNA expression, with or without 100nM norepinephrine stimulation. (B) mRNA expression and (C) protein expression of mitochondrial components. (D) Basal, uncoupled and maximum oxygen consumption rates. (E) mRNA expression of *aP2, Pgc1α, Ucp1* and *Cox8b* in 3T3-F442A adipocytes, after 48 hours treatment of GSK1016790A at indicated doses. Data are presented as mean ± sem. Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, compared to control group.
To investigate the function of TRPV4 in regulating oxidative and thermogenic programs in adipose tissues in vivo, we studied mice with a genetic deletion of Trpv4. These mice are grossly similar to wild-type animals in morphology, behavior and breeding (Liedtke and Friedman, 2003). On a chow diet, their body weight is indistinguishable from WT littermate controls (Figure 2-6 A). In light of the effect of TRPV4 on oxidative metabolism in white adipocytes, we examined gene expression in white adipose tissues from Trpv4-/- and WT control mice.

Subcutaneous adipose tissue has been shown to have a greater thermogenic capacity than other white adipose tissues (Barbatelli et al., 2010) and can significantly contribute to whole body energy homeostasis (Seale et al., 2011). Strikingly, subcutaneous fat from Trpv4-/- mice expressed 30 fold higher Ucp1 mRNA and more UCP1 protein compared to controls (Figure 2-7 A, G). A trend towards increased Pgc1α (p=0.08) and significantly higher Pgc1β were also observed. These mice also have elevated mRNA levels for many genes, including mitochondrial components known to be enriched in BAT, such as Cidea, Cox4il, and Cox8b (Figure 2-7 A).

In general, visceral (epididymal) adipose tissues have a low thermogenic capacity and expresses very little Ucp1 and Cidea (data not shown). Nonetheless, mRNA levels for some BAT enriched genes, such as β3Adr, Pgc1β, CytC, Cox4il and Cox5a, were significantly higher in epididymal fat from the Trpv4-/- mice compared to controls (Figure 2-7 D).
Figure 2-6. *Trpv4*-/- mice gain less body weight on high fat diet. Body weights of male WT and TRPV4-/- mice on chow (A) and HFD (B) over 16 weeks. Data are presented as mean ± sem. (n=9-13 in each group) Comparisons were analyzed by student’s t-test. * P<0.05
Exposure of mice to a high fat diet (HFD) induces obesity and eventually leads to insulin resistance in fed animals. To further understand how TRPV4 deficiency affects gene expression under this metabolic stress, we challenged these mice with a HFD that contains 60% calorie from animal fat. There was no significant body weight difference between the $Trpv4^{-/-}$ and control mice until the animals were on the HFD for 9 weeks (Figure 2-6 B).

The adipose tissues were first examined at 8 weeks of HFD, before the body weight of $Trpv4^{-/-}$ mice diverged from controls. Although the HFD tended to blunt the difference in thermogenic gene expression seen in chow-fed animals, subcutaneous fat from the $Trpv4^{-/-}$ animals nevertheless expressed 3 times higher levels of $Ucp1$ mRNA (Figure 2-7 B) and more UCP1 protein (Figure 2-7 G) than controls. A trend toward higher expression of BAT enriched thermogenic genes such as $\beta 3Adr$, $Pgc1a$ and $Cidea$ was also observed in the $Trpv4^{-/-}$ subcutaneous fat. Histological analysis also showed that mutant mice have smaller fat cells and more UCP1-positive adipocytes in this depot compared to controls (Figure 2-7 H). Despite the low absolute level, significantly higher $\beta 3Adr$ and $Cox8b$ mRNA expression was also observed in the epididymal fat from $Trpv4^{-/-}$ mice (Figure 2-7 E).

As the exposure to the HFD extended to 16 weeks, many BAT enriched and thermogenic genes were no longer different between the $Trpv4^{-/-}$ and control mice, such as $Ucp1$. However, the $Trpv4^{-/-}$ mice still had elevated expression of
Figure 2-7. Altered thermogenic programs in Trpv4-/- adipose tissue. QPCR analysis of mRNA expression of thermogenic and brown fat-selective genes in subcutaneous (A-C) and epididymal (D-F) adipose tissues from Trpv4-/- and WT mice, with exposure to chow (A, D), 8-week high fat diet (B, E) or 16-week high fat diet (C, F). (D) Western blot analysis of UCP1 protein, from chow and 8-week HFD mice. (E) Representative images from immunohistochemistry for UCP1 (brown stain) protein in subcutaneous fat from WT and Trpv4-/- mice after 8 weeks of HFD. UCP1-expressing adipocytes are indicated by arrows. Data are presented as mean ± sem. (n=9-13 in each group) Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, compared to control group.
mRNAs encoding β3adr and Pgc1α in both the inguinal and epididymal fat depots (Figure 2-7 C, F). Overall, in both lean (chow-fed) and obese animals, Trpv4/- mice have more active oxidative and thermogenesis gene expression, which is consistent with our finding in cultured adipocytes in vitro.

**Increased Energy Expenditure Protects TRPV4 Deficient Mice from Diet-induced Obesity**

Based on the gene expression changes observed in Trpv4/- mice, we were interested if the TRPV4 mutation would have a protective role in diet-induced obesity. The body weight curves showed that the TRPV4 mutant mice began to gain significantly less weight after 9 weeks on the HFD, compared to their age- and sex- matched WT littermates (Figure 2-6 B). To determine the exact difference in the components of the weight difference, we first did body composition analysis in Trpv4/- and WT control mice, which showed that the mutant mice had gained less fat, resulting in a higher lean/fat mass ratio compared to WT controls (Figure 2-8 A).

We then performed careful metabolic analysis by indirect calorimetric to determine the cause of the difference in weight gain on high fat diet seen in WT and Trpv4/- mice. Energy expenditure in these mice was measured via oxygen consumption after 8-week of HFD, right before the body weight of mutants diverged from controls. We observed higher oxygen consumption during both day and night time in the Trpv4/- mice, compared to WT controls (Figure 2-8 B), a result consistent with the elevated thermogenic program in white adipose tissue.
Figure 2-8. Increased energy expenditure protects TRPV4 deficient mice from diet-induced obesity. Male WT and Trpv4-/- mice on chow and HFD over 16 weeks. (A) MRI analysis of body composition (fat, lean and water mass) after 12 weeks HFD. energy expenditure (as oxygen consumption rate, B and CO2 production rate, C), 24-hour food intake (D) and physical activity (E) was measured in individually housed WT and Trpv4-/- mice after 8 weeks HFD. Data are presented as mean ± sem. (n=9-13 in each group) Two-way ANOVA was used for panel B, C and E; others single comparisons were analyzed by student’s t-test. * P<0.05, ** P<0.01, *** P<0.001, n.s. not significant.
A higher CO2 production was observed in Trpv4-/- mice (Figure 2-8 C). Importantly, there was no significant difference in food intake (Figure 2-8 D) or physical activity (Figure 2-8 E) between the two genotypes, indicating the energy intake or the expenditure by physical movement was not altered in the Trpv4-/- mice. Taken together, these data strongly suggest that the reduced weight gain upon HFD in Trpv4-/- mice was due to, at least in part, an increased energy expenditure associated with increased thermogenesis in their white adipose tissue.

**A Cell-autonomous Up-regulation of Thermogenic Program in Trpv4-/- Adipocytes**

We observed the expected physiological changes in the Trpv4-/- mice according to their adipose gene expression changes. However, because the Trpv4-/- mice we studied have a whole body TRPV4 deficiency, it is important to know whether any other metabolically active tissues also contribute to the whole body phenotype; and if so, how much of the whole body phenotype was due to the “browning” of the white adipose tissue and how much was coming from other tissues.

We first examined the classic brown adipose tissue, the interscapular BAT, in Trpv4-/- and WT control mice. Under both chow (Figure 2-9 A) and HFD conditions (Figure 2-9 B), no significant difference in mRNA expression of thermogenic or oxidative genes was detected between the mutant and WT mice.
Figure 2-9. *Trpv4*/*- mice have minimal change in thermogenic and oxidative pathways in interscapular brown fat and skeletal muscle. The expression of thermogenic and brown fat specific genes were examined by QPCR in interscapular brown fat from *Trpv4*/*- and WT mice, under chow (A) or 16-week high fat diet (B). Expression of genes involved in oxidative metabolism were examined from quadriceps muscle from *Trpv4*/*- and WT mice, under chow (C) or 16-week high fat diet (D) conditions. Data are presented as mean ± sem. Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, n.s. not significant, compared to control group.
There was a report that TRPV4 deficiency caused a higher oxidative capacity in skeletal muscle (Kusudo et al., 2011). However, in that report, the authors only examined the soleus muscle in mice. More importantly, they only assessed the oxidative gene expression in the soleus muscle after a significant difference in body weight had occurred between knockout and WT mice. We examined quadriceps gene expression of Trpv4-/- and WT mice under chow and HFD. While myogenin expression were up-regulated in the mutant mice under HFD as reported (Figure 2-9 D), there was no difference seen in myogenin as well as the oxidative/mitochondrial genes, such as carnitine palmitoyltransferase I- beta (Cpt1b), citrate synthase (Cs), cytochrome C (CytC) and Isocitrate dehydrogenase (Idh3a), when mice were on chow diet and lean (Figure 2-9 C). This suggested that the difference observed in the other report was likely a secondary effect of obesity.

We examined two metabolically active organs, BAT and skeletal muscle. Neither of them seemed to have significant gene expression changes associated with TRPV4 deficiency, whereas strong effects were seen in the white adipose tissue. However, it is still not clear whether the difference in white adipose tissue was due to secondary effects from other tissues that we did not look at, such as brain, sensory neurons, etc. Therefore we asked if the phenotype observed in vivo was associated with any cell-autonomous alterations in adipocyte cultures derived from these mice. To test this, stromal-vascular cells from the adipose tissue of young, lean Trpv4 -/- and WT mice were isolated and stimulated to differentiate
Figure 2-10. TRPV4 controls adipocyte thermogenic gene program in a cell-autonomous manner. (A) Mitochondrial and brown fat selective gene expression in *in vitro* differentiated *Trpv4-/-* and WT primary adipocytes at the basal level. (B) *Pgc1a* and *Ucp1* mRNA in these adipocytes at basal, and after stimulation with 10nM or 100nM norepinephrine for 4 hours. Data are presented as mean ± sem. Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, n.s. not significant, compared to control group.
into adipocytes \textit{in vitro}. After 8 days, greater than 90\% of the cells were fully differentiated. Compared to those from WT controls, unstimulated adipocytes from \textit{Trpv4-/-} animals showed elevated mRNA expression for \textit{Pgc1a} and \textit{Ucp1}, \textit{\beta3adr}, \textit{Cox7a}, \textit{Cox8b}, \textit{CytC} and \textit{Cidea} (Figure 2-10 A). Importantly, when stimulated with 10nM or 100nM norepinephrine, the \textit{Trpv4-/-} adipocytes had much greater responses in terms of \textit{Pgc1a} and \textit{Ucp1} expression (Figure 2-10 B). Taken together, these data indicate that TRPV4 controls the oxidative and thermogenic programs in a cell autonomous manner.
Discussion

In this chapter, we identified a novel connection between the TRPV channels and the regulation of PGC1α expression, by a high-throughput, quantitative PCR based chemical screen. The initial candidate from the primary screen turned out to be an off-target effect of a well-characterized drug-like compound. We identified the actual target of this compound through both pharmacological and genetic approaches. We demonstrated that functional TRPV4 channel was expressed in cultured adipocytes *in vitro* and in adipose tissues, particularly white adipose tissue *in vivo*. Genetic or pharmacological manipulation of TRPV4 clearly demonstrated that TRPV4 was a negative regulator of PGC1α and the downstream gene programs controlled by PGC1α, namely the mitochondrial oxidative program and thermogenesis. A mouse model with a genetic TRPV4 deficiency showed consistent phenotypes in terms of gene expression and physiology, according to the functions of TRPV4 identified *in vitro*.

The High Throughput Screen Platform for Mature Adipocytes

We modified and optimized the previously used QPCR-based chemical screen platform for adipocytes. It is of particular interest to study mature adipocytes, especially given the relevance in obesity and metabolic diseases. However, one technical challenge of performing high throughput screens is that there is a large amount of lipid in fully differentiated adipocytes that often interferes with the micropipettes handling liquids throughout the steps. One simple solution is to lower the cell density in such assays. However, cell confluence is usually
required for good \textit{in vitro} differentiation in fat cell lines or primary adipocytes. In our optimized system, we balanced the cellular density and lipid content by trypinizing and re-plating “half-differentiated” adipocytes into high throughput format (384 wells) two days after the initiation of differentiation. This method resulted in a good yields and highly consistent performance. We have used this platform for screening for inducers of PGC1\(\alpha\) mRNA expression, but it is conceivable that it can be easily modified for other expression based or image based screening in mature adipocytes.

**TRPVs and Adipocyte Biology**

One advantage of using a library consisting of compounds mostly with known bioactivity is to identify novel connections between a well characterized pathway and the screening target. TRPVs are a well-characterized ion channel family, particularly in terms of their channel electrophysiology and biophysical properties. Most detailed information about the TRPV family came from the study of TRPV1, the capsaicin and putative heat receptor in sensory neurons. Extensive and intensive pharmacology has been done on the TRPV1 channel, with a goal of developing new classes of peripheral acting analgesics. Many small molecules (both agonist and antagonist) targeting TRPV1 or other TRPVs have been developed and some of them have been used in clinical trials (Wong and Gavva, 2009).

The sophisticated pharmacology of TRPVs makes the identification of the new, cell-autonomous functions of TRPVs in regulating adipocyte physiology
particularly interesting. There have been reports suggesting TRPVs might play roles in different aspects of adipose biology. TRPV1 has been reported to inhibit adipogenesis \textit{in vitro} and \textit{in vivo}. Paradoxically, both TRPV1 deficiency and activation were suggested to protect animals against diet induced obesity, although the mechanism and/or responsible cell/tissue types have not been well understood (Motter and Ahern, 2008; Zhang et al., 2007). Recently, TRPM8 also has been suggested to regulate thermogenesis in classical brown adipose tissue, but it is unclear if the effect was cell-autonomous or through other CNS mediated mechanisms (Ma et al., 2012). Nevertheless, this study first demonstrated that a chemically trackable TRPV channel can regulate energy metabolism in adipocytes in a cell-autonomous manner, both in cultured cells and in whole animals. More importantly, this regulation appeared to play an important role in the development of obesity and pathogenesis of metabolic disorders. This finding illustrated that chemical biology approaches are not only extremely powerful in identifying novel pathway, but could also bring the finding quickly to pharmacology and potential therapeutic applications.
Materials and Methods

Materials

Antibody sources are as follows: anti-UCP1 and anti-OXPHOS (Abcam), anti-TRPV4 (Alomone). Forskolin, norepinephrine, GSK1016790A, AMG9810, AM251, insulin, dexamethasone, isobutylmethylxanthine and puromycin were from Sigma. BCTC was from Tocris. SLV319, CAY10508 and Rosiglitazone were from Cayman Chemica. shRNA constructs were in pLKO vectors (for lentivirus) or pMKO vectors (for retrovirus).

Animals

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee of Dana-Farber Cancer Institute. Mice were either maintained on a standard rodent chow or a 60% high-fat diet (Research Diets) with 12-hour light and dark cycles. Trpv4-/- mice were provided by Dr. Liedtke (Liedtke and Friedman, 2003) and back-crossed to C57BL/6J (Jackson Lab) for 10 generations before all studies. Each study group contains 9-13 animals of each genotype.

Chemical Screen

Briefly, after two days of differentiation, 3T3-F442A adipocytes were trypsinized and split into 384 well plates (3000 cell/well). At day 6, adipocytes were treated with a chemical library (Broad Institute) in 384-well plates for 20 hours at ~20uM. mRNA was harvested using the TurboCapture kit (Qiagen), reverse transcribed
to cDNA, and quantified by qPCR with Sybr-Green (ABI). All values were normalized to vehicle (DMSO) treated wells.

**Cell Culture**

For virus production, 293T (for lentivirus) or ϕnx cells (retrovirus) were transfected with Fugene 6 (Roche) with viral vectors. Viral supernatant was harvested 48 hours later. 3T3-F442A pre-adipocytes were infected for 4 hour (lenti) or overnight (retro), followed by puromycin selection (2ug/ml). 3T3-F442A adipocyte differentiation was induced in cultures by treating confluent cells with 850 nM insulin for 8-10 days. To stimulate thermogenesis, cells were incubated with forskolin (10uM) or norepinephrine (10nM or 100nM) for 4 hours. For primary adipocytes, SVF from inguinal fat depots of 5-week-old male mice were prepared and differentiated for 8 days as previously described (Kajimura et al., 2009). Rosiglitazone (1uM) was used for the first two days of differentiation. n=3 or 4 in each group for all cell culture experiments.

**QPCR and Western Blotting**

Total RNA from cultured cells or tissues was isolated using the TRIzol method (Invitrogen) combined with Qiagen RNAEasy mini columns according to the manufacturer’s instruction. For qPCR analysis, RNA was reverse transcribed using the ABI high capacity cDNA synthesis kit and used in quantitative PCR reactions containing SYBR-green fluorescent dye (ABI). Relative expression of mRNAs was determined after normalization with TBP levels using the ∆∆Ct method. For TRPV1, TRPV2 and TRPV4 expression, the standard curve method
was used to quantify the absolute copy numbers. The standard curves were generated with pMSCV plasmids containing TRPV1, TRPV2 and TRPV4 cDNA. For Western blot analysis, cells or tissues were lysed in RIPA buffer (0.5% NP-40, 0.1%sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5). Lysates were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), and probed with the indicated antibodies.

**Calcium Imaging**

At day 6 of differentiated, 3T3-F442A adipocytes were trypsinized and transferred to coverslip coated with Cell-Tak solution (BD Biosciences). Cells were loaded with 10 uM Fura-2AM (Invitrogen) for 20 min at 37°C and then washed twice in standard Tyrodes Solution (in mM): 135 NaCl, 4 KCl, 10 glucose, 10 HEPES, 1.2 CaCl2, 1 MgCl2, pH 7.40 at room temperature. Fluorescence images were obtained (at 510 nm) using an Olympus IX81 inverted microscope with a 20X objective (Olympus) and a CCD camera (Hamamatsu, Model# C4742-80-12AG) upon sequential excitation with 340 nm followed by 380 nm light. After establishing a baseline 340/380 ratio, a selective TRPV4 agonist, GSK101 (100nM) was perfused onto the cells.

**Oxygen consumption assays**

3T3-F442A adipocytes expression control shGFP retrovirus or sh-TRPV4 were induced to undergo adipogenesis. At day 8 of differentiation, oxygen consumption was measured in fat cells using a strathkelvin Clark-type electrode.
1 μM oligomycin (Sigma-Aldrich) or 5uM FCCP (Sigma-Aldrich) was added to block state III respiration and induce uncoupling, respectively.

**Metabolic Study**

Body weight was measured on a weekly basis for high-fat feeding experiments. Fat and fat-free mass was measured by MRI. Whole-body energy metabolism was evaluated using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbia Instruments). Mice were acclimated in the metabolic chambers for 2 days before the starting the experiment to minimize stress from the housing change. CO2 and O2 levels were collected every 32 minutes for each mouse over a period of 3 days. Movement and food intake are measured more frequently at regular intervals.

**Histological analysis**

Tissues were dissected and fixed in 4% paraformaldehyde overnight and rinsed with phosphate-buffered saline before embedding in paraffin. For Ucp1 immunohistochemistry, paraffin embedded sections were incubated with anti-Ucp1 (Abcam), followed by detection using the ABC Vectastain-Elite kit (Vector Labs) according to the manufacturer’s instructions.
Chapter 3:

TRPV4 Regulates Pro-inflammatory Pathways in Adipocytes
Introduction

To understand the function of TRPV4 in adipocytes more fully, microarray analysis of global gene expression was performed with mRNA from 3T3-F442A adipocytes expressing shRNAs against TRPV4 or GFP. As expected, many genes whose expression was strongly increased were involved in brown adipocyte function and oxidative metabolism (Table 1). For example, acetyl-coenzyme acyltransferase 1B, an enzyme mediating the last step of fatty acid β-oxidation, was expressed 5 fold higher in TRPV4-knockdown adipocytes compared to controls. Similarly, mRNAs encoding the β-3 and β-1 adrenergic receptors, key receptors for catecholamines that control brown adipocyte function, were increased more than 3 fold. Pgc1α was also one of the most highly regulated genes on these arrays.

Strikingly, many genes whose expression was decreased by TRPV4 knockdown were chemokines/cytokines or genes related to pro-inflammatory pathways (Table 1). For example, in the array the chemokine Ccl7 (Mcp3) mRNA was decreased by more than 85% in TRPV4 knockdown adipocytes. Expression of mRNA encoding Saa3, a pro-inflammatory amyloid protein secreted from adipose tissue, was reduced by 98%.

This unexpected finding of TRPV4’s role in pro-inflammatory gene expression is particularly interesting, because chronic, low-grade inflammation in adipose tissue has been shown to be a hallmark of obesity and thought to be a major
Table 1. Genes regulated by TRPV4-knockdown in 3T3-F442A adipocytes

<table>
<thead>
<tr>
<th>Up-regulated Genes</th>
<th>Change</th>
<th>Down-regulated Genes</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>similar to hepatocellular carcinoma-associated gene TD26</td>
<td>18.54</td>
<td>serum amyloid A 3</td>
<td>-49.82</td>
</tr>
<tr>
<td>thyroid hormone responsive SPOT14 homolog (Rattus)</td>
<td>13.34</td>
<td>interferon-induced protein 44</td>
<td>-45.15</td>
</tr>
<tr>
<td>gastrin releasing peptide receptor</td>
<td>7.46</td>
<td>chemokine (C-X-C motif) ligand 10</td>
<td>-25.11</td>
</tr>
<tr>
<td>carbonic anhydrase 9</td>
<td>6.66</td>
<td>2’-5’ oligoadenylate synthetase-like 2</td>
<td>-17.5</td>
</tr>
<tr>
<td>sulfotransferase family 1A, phenol-preferring, member 1</td>
<td>5.42</td>
<td>lipocalin 2</td>
<td>-17.44</td>
</tr>
<tr>
<td>RAR-related orphan receptor gamma</td>
<td>5.07</td>
<td>interferon activated gene 203</td>
<td>-11.88</td>
</tr>
<tr>
<td>aldehyde dehydrogenase family 1, subfamily A1</td>
<td>4.99</td>
<td>interferon-induced protein with tetrasaccharide repeats 1</td>
<td>-11.42</td>
</tr>
<tr>
<td>phosphorylase kinase gamma 1</td>
<td>4.99</td>
<td>lipopolysaccharide binding protein</td>
<td>-11.32</td>
</tr>
<tr>
<td>acetyl-Coenzyme A acyltransferase 1B</td>
<td>4.9</td>
<td>2’-5’ oligoadenylate synthetase 1A</td>
<td>-9.84</td>
</tr>
<tr>
<td>proteinase 3</td>
<td>4.82</td>
<td>interleukin 6</td>
<td>-9.35</td>
</tr>
<tr>
<td>mannan-binding lectin serine peptidase 1</td>
<td>4.79</td>
<td>toll-like receptor 2</td>
<td>-9.26</td>
</tr>
<tr>
<td>thyroid hormone responsive SPOT14 homolog (Rattus)</td>
<td>4.79</td>
<td>chemokine (C-X-C motif) ligand 5</td>
<td>-8.47</td>
</tr>
<tr>
<td>Rho GTPase activating protein 6</td>
<td>4.77</td>
<td>secreted frizzled-related protein 2</td>
<td>-7.48</td>
</tr>
<tr>
<td>Inhibitor of DNA binding 4</td>
<td>4.68</td>
<td>phosphoenolpyruvate carboxykinase 1, cytosolic</td>
<td>-7.28</td>
</tr>
<tr>
<td>growth hormone releasing hormone</td>
<td>4.53</td>
<td>inhibin beta-A</td>
<td>-6.98</td>
</tr>
<tr>
<td>selenium binding protein 1</td>
<td>4.47</td>
<td>complement factor B</td>
<td>-6.86</td>
</tr>
<tr>
<td>selenium binding protein 1</td>
<td>4.37</td>
<td>receptor transporter protein 4</td>
<td>-6.83</td>
</tr>
<tr>
<td>adrenergic receptor, beta 1</td>
<td>4.33</td>
<td>chemokine (C-X-C motif) ligand 7</td>
<td>-6.63</td>
</tr>
<tr>
<td>hippocalcin</td>
<td>4.22</td>
<td>insulin-like growth factor binding protein 3</td>
<td>-6.57</td>
</tr>
<tr>
<td>complement factor D (adipsin)</td>
<td>4.17</td>
<td>STEAP family member 4</td>
<td>-6.43</td>
</tr>
<tr>
<td>Mannan-binding lectin serine peptidase 1</td>
<td>4.11</td>
<td>WNT1 inducible signaling pathway protein 1</td>
<td>-6.41</td>
</tr>
<tr>
<td>phosphorylase kinase gamma 1</td>
<td>3.99</td>
<td>chemokine (C-X-C motif) ligand 1</td>
<td>-6.32</td>
</tr>
<tr>
<td>inhibitor of DNA binding 4</td>
<td>3.88</td>
<td>interferon activated gene 203</td>
<td>-6.23</td>
</tr>
<tr>
<td>WNK lysine deficient protein kinase 4</td>
<td>3.84</td>
<td>cytochrome P450, family 7, subfamily b, polypeptide 1</td>
<td>-6.18</td>
</tr>
<tr>
<td>inhibitor of DNA binding 4</td>
<td>3.78</td>
<td>calpain 6</td>
<td>-6.13</td>
</tr>
<tr>
<td>cytochrome c oxidase, subunit VIIIb</td>
<td>3.77</td>
<td>lumican</td>
<td>-6.1</td>
</tr>
<tr>
<td>resistin</td>
<td>3.73</td>
<td>solute carrier family 15, member 3</td>
<td>-6.06</td>
</tr>
<tr>
<td>epoxide hydrolase 2, cytoplasmic</td>
<td>3.71</td>
<td>runt related transcription factor 1</td>
<td>-5.97</td>
</tr>
<tr>
<td>inhibitor of DNA binding 4</td>
<td>3.64</td>
<td>runt related transcription factor 1</td>
<td>-5.91</td>
</tr>
<tr>
<td>adrenergic receptor, beta 3</td>
<td>3.61</td>
<td>interleukin 1 receptor-like 1</td>
<td>-5.83</td>
</tr>
<tr>
<td>apoptosis-associated tyrosine kinase</td>
<td>3.6</td>
<td>interferon, alpha-inducible protein 27</td>
<td>-5.74</td>
</tr>
<tr>
<td>adrenergic receptor, beta 3</td>
<td>3.57</td>
<td>component of Sp100-rs /// similar to component of Sp100-rs</td>
<td>-5.44</td>
</tr>
<tr>
<td>peroxisome proliferative activated receptor, gamma, coactivator 1 alpha</td>
<td>3.54</td>
<td>ribonuclease, RNase A family 4</td>
<td>-5.33</td>
</tr>
<tr>
<td>gastrin releasing peptide receptor</td>
<td>3.49</td>
<td>interferon inducible GTPase 1</td>
<td>-5.29</td>
</tr>
<tr>
<td>Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha</td>
<td>3.39</td>
<td>P lysozyme structural</td>
<td>-5.26</td>
</tr>
</tbody>
</table>

Table 1. Positive fold change means higher expression in adipocytes with shRNA against TRPV4 compared to controls (shGFP). Negative value means reduced expression in these cells. Values in the table represented means from two samples in each group.
cause of insulin resistance associated with obesity (Gregor and Hotamisligil, 2011). The initial discovery of this connection between chronic inflammation and insulin resistance was made more than 20 years ago. Since then, numerous studies have demonstrated that this inflammation was critical for the development of insulin resistance associated with obesity.

The broad and substantial reduction of the chemotactic gene program in TRPV4 knockdown adipocytes, rather than changes in individual genes, indicated that TRPV4 might function as an unexpected, yet very important upstream controller of this program. If true, manipulating TRPV4 would likely have profound effects in the expression and secretion of chemokines, and would therefore affect the recruitment of immune cells, with attendant metabolic consequences.
Results

TRPV4 Positively Controls a Pro-inflammatory Gene Program

Based on the microarray results, we used qPCR to further analyze the expression of 22 genes that are either highly regulated by TRPV4 (from the array) or are known from published literature to be important in adipose inflammation. Importantly, experimental reduction of TRPV4 expression had a profound inhibitory effect on a whole array of chemokines, such as Ccl2 (Mcp1), Ccl3 (Mip1α), Ccl5 (Rantes), Ccl7 (Mcp3), Cxcl1 (KC), Ccl8, Cxcl5 and Cxcl10 and cytokines such as Il6, Saa3 and Thrombospondin (Figure 3-1 A). A similar effect was observed on the expression of other genes important for inflammatory processes, such as Tlr2, Timp1, Socs3, Socs5, Mmp2, Fas and Vcam (Figure 3-1 B).

Conversely, mRNA expression of Mip1α, Cxcl1, Il6, Timp1 and Tlr2, can be induced by treating adipocytes with the TRPV4 agonist (Figure 3-2 A). This effect is specific and dependent on TRPV4, as shRNA against TRPV4 fully abolished the induction by the agonist (Figure 3-2 A).

To determine if these effects on gene expression resulted in alterations in chemokine secretion from adipocytes, we measured levels of secreted MCP1, MIP1α, CXCL1 and RANTES in culture medium by ELISA. Similar to what we observed at the mRNA level, the concentrations of MCP1, CXCL1 and RANTES were each reduced by more than 85% in the culture medium from the TRPV4 knockdown adipocytes, compared to controls (Figure 3-2 B). The TRPV4 agonist
Figure 3-1. Loss of TRPV4 reduces pro-inflammatory gene expression in adipocytes. QPCR analysis of mRNA encoding chemokines/cytokines (A) and other genes involved in inflammatory pathways (B) in 3T3-F442A adipocytes with retrovirus expressing either shTRPV4 or shGFP (control).
induced MIP1α protein by 76 fold. Again, this induction was fully abolished by
knocking down TRPV4 (Figure 3-2 B). These data indicate a very powerful role
for TRPV4 in the regulation of a pro-inflammatory pathway in adipocytes.

**TRPV4 Deficiency Results in Reduced Pro-inflammatory Gene Expression**

*in vivo*

We studied *Trpv4*-/- mice as described in Chapter 1, to examine the *in vivo*
function of TRPV4 on the pro-inflammatory program. We measured the
expression of pro-inflammatory genes, especially chemokines, identified from the
analysis of TRPV4 knockdown 3T3-F442A adipocytes. These included *Mcp1*,
*Mip1α*, *Mcp3*, *Rantes* and *Vcam*. These genes were expressed at very low levels
in the adipose tissues of lean animals, and no significant differences were
observed in either subcutaneous or epididymal adipose tissues between the
mutants and controls on a chow diet (Figure 3-3 A, B).

Exposure of wild type mice to a high fat diet (HFD) effectively induces obesity
and provokes adipose inflammation, eventually contributing to insulin resistance.
To further understand how TRPV4 deficiency affects adipose inflammation under
this metabolic stress, we challenged *Trpv4*-/- mice and wild type controls with a
60% high fat diet.

Being aware that *Trpv4*-/- mice would gain less weight than WT controls, we
wanted to eliminate the possible confounding effects from the body weight
difference. Therefore, adipose tissues were first examined at 8 weeks of HFD,
before the body weight of *Trpv4*-/- mice diverged from controls. We first looked at
Figure 3-2. Activation of TRPV4 induces pro-inflammatory gene expression and protein secretion. (A) mRNA expression of *Mcp1*, *Mip1α*, *Rantes*, *Mcp3*, *Il6*, *Cxcl1*, *Timp1* and *Tlr2* in 3T3-F442A adipocytes with shTRPV4 or shGFP, with or without 48 hours agonist treatment. (B) Protein concentrations of MCP1, MIP1α, CXCL1 and RANTES in culture medium from cell in (A) were determined by ELISA. Data are presented as mean ± sem. Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, compared to control group.
visceral fat (epididymal fat) as this depot has more inflammation and is the primary source of inflammatory cytokine/adipokines. As expected, eight weeks of HFD was enough to significantly elevate the mRNA expression of many chemokines in epididymal fat in WT mice, such as \( \text{Mcp1} \) (5 fold), \( \text{Mip1}\alpha \) (13 fold), \( \text{Rantes} \) (2 fold) and \( \text{Mcp3} \) (28 fold), compared to animals on a chow diet.

Interestingly, without a significant difference in total adiposity, \( \text{Trpv4-/-} \) mice showed a substantial decrease in the mRNA expression of \( \text{Mcp1} \) (40%), \( \text{Mip1}\alpha \) and \( \text{Mcp3} \) (50%), relative to controls (Figure 3-3 A). Similarly, the induction of those genes in the subcutaneous fat in WT mice in response to HFD was also largely blunted in the \( \text{Trpv4-/-} \) mice (Figure 3-3 B).

At 16 weeks of the HFD, compared to chow-fed animals, chemokine gene expression in epididymal fat continued to rise in WT mice: \( \text{Mcp1} \) (10 fold), \( \text{Mip1}\alpha \) (72 fold), \( \text{Rantes} \) (4 fold) and \( \text{Mcp3} \) (47 fold). The expression of mRNAs for \( \text{Mip1}\alpha \) and \( \text{Vcam} \) remained low (reduced by 70% and 30%) in the \( \text{Trpv4-/-} \) mice, compared to WT controls; the differences in \( \text{Mcp1} \) and \( \text{Mcp3} \) were blunted (Figure 3-3 A). Similar differences were observed in the inguinal adipose tissues (Figure 3-3 B).

\textit{Trpv4-/- Mice Have Reduced Inflammation in Adipose Tissue and Improved Glucose Tolerance}

Obesity is associated with chronic “metainflammation” in adipose tissue (Hotamisligil, 2006). Cytokines such as TNF\( \alpha \) (Hotamisligil et al., 1995; Hotamisligil et al., 1993) and IL-1\( \beta \) (Lagathu et al., 2006) are secreted from
Figure 3.3. Altered pro-inflammatory programs in \textit{Trpv4}-/- adipose tissue. mRNA expression of chemokine/chemoattractant genes in epididymal (A) and subcutaneous (B) fat from WT and \textit{Trpv4}-/- were analyzed by QPCR, under all three diet conditions. Data are presented as mean ± sem. (n=9-13 in each group) Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, compared to control group.
immune cells in the inflamed adipose tissue (Weisberg et al., 2003; Xu et al., 2003), and are believed to be a major contributor to systematic insulin resistance.

To understand the biological impact of the changes in chemokine gene expression caused by TRPV4 deficiency (Figure 3-3 AB), we analyzed the expression of macrophage selective markers (F4/80, CD68 and CD11b) to quantify macrophage infiltration in Trpv4-/- and WT epididymal fat from all three diet groups: chow, 8-week HFD and 16-week HFD. We were interested to see if reduced chemotactic gene expression would result in decreased macrophage infiltration. As expected, HFD increased the expression of all three macrophage markers in WT adipose tissue (5-10 fold increased by 8 weeks, 10-30 fold increased by 16 weeks) (Figure 3-4 A), indicating that macrophages have been actively recruited into adipose tissue in response to high fat diet. Consistent with the reduction in chemokine expression, Trpv4-/- adipose tissue showed a 40% or 60% reduction in the expression of all three macrophage markers mRNA after 8 or 16 weeks of HFD, respectively (Figure 3-4 A). This suggests that there were significantly fewer macrophages being recruited into Trpv4-/- adipose tissue compared to WT controls. Indeed, histologic analysis also showed there were far fewer “crown-like-structures”, previously shown to represent macrophages in fat tissues (Cinti et al., 2005), in the Trpv4-/- epididymal fat compared to WT controls (Figure 3-4 B).

Macrophages have been shown to be the major source of TNF-α and other inflammatory cytokines in inflamed adipose tissue. To further assess the inflammation associated with decreased macrophage infiltration, the mRNA
Figure 3-4. *Trpv4*-/- adipose tissue have less macrophage infiltration. mRNA expression of three macrophage markers in epididymal fat from WT and *Trpv4*-/- mice on chow, 8-week HFD and 16-week HFD (A). H&E staining of epididymal adipose tissues from WT and *Trpv4*-/- mice after 16-week HFD (B), arrows indicates “crown like structures” (CLS) consisting of macrophages. Data are presented as mean ± sem. (n=9-13 in each group) Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, compared to control group.
expression of Tnfa, a key cytokine for obesity-induced insulin resistance was measured. HFD significantly increased Tnfa mRNA in WT adipose tissue, while the induction was reduced by more than 30%-40% in adipose tissue from Trpv4-/- mice (8 weeks HFD or 16 weeks HFD, respectively) (Figure 3-5 A).

Furthermore, phosphorylation of serine\(^{273}\) on PPAR\(\gamma\), a recently identified modification that is associated with obesity and insulin resistance (Choi et al., 2010), was substantially attenuated in the Trpv4 -/- adipose tissue compared to WT controls (Figure 3-5 B) after 8 weeks or 16 weeks of high fat diet, strongly suggested that there was less inflammation and likely enhanced insulin sensitivity in the Trpv4-/- adipose tissue.

Adipose tissue inflammation is associated with insulin resistance and could be represented as glucose intolerance in a standard glucose tolerance test. To assess if the reduction in TNF\(\alpha\) and PPAR\(\gamma\) phosphorylation has a physiological effect on insulin sensitivity, intraperitoneal glucose tolerance tests were first performed 7 weeks after HFD, when no difference in body weight had developed between the two genotypes. As shown in Figure 3-5 C, Trpv4-/- mice showed a small yet significant improvement in glucose tolerance as early as 7 weeks after high fat diet. As these mice continued on the diet (12 weeks), the relative improvement in glucose tolerance of mutants compared to controls became more apparent (Figure 3-5 D).
Figure 3-5. *Trpv4*−/− mice have less inflammation and improved glucose tolerance. mRNA expression of *Tnfa* (A) in epididymal fat from WT and *Trpv4*−/− mice on chow, 8-week HFD and 16-week HFD. (B) Western blot analysis of PPARγ serine-273 phosphorylation and total PPARγ in epididymal fat after 8-week and 16-week HFD. Glucose tolerance tests, blood glucose levels were measured in 7 weeks (C) or 12 weeks (D) high fat-fed WT or *Trpv4*−/− mice, after overnight fasting (time 0) and at the indicated times after intraperitoneal injection of glucose (1.5g/kg body weight for 7W-HFD and 1g/kg body for 12w-HFD). Data are presented as mean ± sem. (n=9-13 in each group) Two-way ANOVA was used for panel A, C, and D; others single comparisons were analyzed by student’s t-test. * P<0.05, ** P<0.01, *** P<0.001, n.s. not significant.
TRPV4 Deficiency Affects Adipocyte Pro-inflammatory Gene Program in a Cell-autonomous Manner

Again, since Trpv4 -/- mice have whole-body TRPV4 deficiency, we asked if the phenotype observed in vivo was associated with cell-autonomous alterations in adipocyte cultures derived from these mice. To examine this, stromal-vascular cells from the adipose tissue of young, lean Trpv4 -/- and WT mice were isolated and stimulated to differentiate into adipocytes in vitro. After 8 days, greater than 90% of the cells were fully differentiated. Importantly, the mRNA expression of pro-inflammatory chemokine/cytokines, such as Mcp1, Mip1α, Mcp3, Tnfα and Vcam were reduced by more than 80% in Trpv4-/- primary adipocytes (Figure 3-6 A).

Macrophages share many aspects of gene regulation with adipocytes. It is common that one molecule that regulates metabolic and/or inflammatory pathways in one cell type also regulates the same pathways in the other (Hotamisligil, 2006). Indeed, many of the chemokines we identified to be regulated by TRPV4 could also be secreted from macrophages, probably to a greater extent than they are secreted from the adipocytes. Moreover, in the case of pro-inflammatory signaling and immune cell recruitment, because macrophages are the cells that directly respond to the secreted chemokines, it is especially critical to know if macrophages from the Trpv4-/- background have compromised pro-inflammatory secretion and/or response to those signals.
Figure 3-6. TRPV4 controls adipocyte pro-inflammatory gene program in a cell-autonomous manner. (A) Chemokines and Tnfa mRNA expression in \textit{in vitro} differentiated \textit{Trpv4-/-} and WT primary adipocytes at the basal level. (B) Chemokines and Tnfa mRNA expression in peritoneal macrophages isolated from \textit{Trpv4-/-} and WT animals. (C) Chemokines and Tnfa mRNA expression in bone marrow derived \textit{Trpv4-/-} and WT macrophages, at basal and LPS stimulated levels. (C) Chemokines and Tnfa mRNA expression in bone marrow derived \textit{Trpv4-/-} and WT macrophages, at basal and free fatty acid stimulated levels.
Primary peritoneal macrophages were first isolated from WT and Trpv4-/- mice. Their gene expression was examined as the basal tone of macrophage pro-inflammatory program. In contrast to the dramatic difference we observed in primary adipocytes, no significant difference in mRNA expression of chemokines (Mcp1, Mip1a, Rantes, Mcp3 and Vcam) or inflammatory cytokines such as Tnfa was observed between the macrophages from the two genotypes (Figure 3-6 B).

To further examine the function of macrophages beyond the basal stage, we tested whether TRPV4 deficient macrophages could response normally to pro-inflammatory stimulation, such as the typical M1 stimulation by LPS. We were also interested in stimulation with free fatty acids, which have been shown to be an important inflammatory signal in the context of obesity and diabetes. To do so, we derived macrophages from bone marrow precursors from WT and Trpv4-/- mice. In vitro differentiated macrophages were then stimulated with either LPS or FFA. As expected, LSP potently increased the expression of Mip1a (50 fold), Mcp3 (20 fold), Vcam (20 fold) as well as Tnfa (50 fold) mRNA in WT and Trpv4-/- macrophages without significant difference (Figure 3-6 C), except there was a small decrease in Mcp1 induction observed in mutant macrophages.

In the same time, free fatty acid (palmitate) also significantly induced the expression of these genes, although to a lesser extent (2-10 fold) compared to LPS treatment. Again, no significant difference in terms of responses was observed between two genotypes; expect a small difference in basal Tnfa expression (Figure 3-6 D).
Taken together, these data indicate that TRPV4 controls pro-inflammatory gene programs in a cell autonomous manner in adipocytes, but does not appear to significantly affect the same pathway to macrophages.

**Pharmacological Inhibition of TRPV4 Represses the Pro-inflammatory Program and Improves Insulin Resistance**

We have demonstrated a negative role of TRPV4 in regulating pro-inflammatory program in white adipocytes *in vivo* and *in vitro*, mostly using genetic approaches. It is interesting to investigate if pharmacological tools that inhibit TRPV4 activity would therefore repress these pathways. This would be particularly useful as these kinds of agents could potentially be used *in vivo* to attenuate obesity-related disorders.

Compared to the other member of the TRPV family TRPV1, there are few specific antagonists available for TRPV4. GSK205 was reported to be a TRPV4 specific antagonist with an IC50 around 1uM (Phan et al., 2009). We treated fully differentiated F442A adipocytes with 5uM GSK205 for 48 hours. While the fat differentiation was not altered *per se*, the antagonist treatment significantly suppressed pro-inflammatory genes such as *Mcp1, Mip1a, Rantes* and *Mcp3* (Figure 3-7 A) in adipocytes.
Figure 3-7. TRPV4 antagonist GSK205 represses pro-inflammatory gene expression and improves insulin resistance. 3T3-F442A adipocytes were treated with 10uM GSK205 or DMSO for 48 hours before mRNA expression of adipogenesis marker AP2 and pro-inflammatory chemokines were analyzed by QPCR (A). 7 days of B.I.D. 10mg/kg GSK205 by intraperitoneal injection did not significantly affect body weight of C57/B6 mice that have been on high fat diet for 14 weeks (B). GSK205 treated HFD mice have reduced expression of chemokines and *Tnfa* mRNA in epididymal fat (C) and improved glucose tolerance (D). Data are presented as mean ± sem. (n=9-13 in each group) Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, compared to control group.
We were interested in testing whether using GSK205 in vivo could affect adipocyte gene expression and function. This is also a complex problem because many aspects of this compound could affect the interpretation of the results, such as the pharmacological kinetics, pharmacological dynamics, and the hydrophobicity of the compound. Preliminary pharmacological kinetics studies indicated that GSK205 has a half-life of 2 hours after IP injection in mice plasma (unpublished result from communication with Dr. Patrick Griffin). Although the PK is unfavorable for long term treatment, as a proof-of-principle study, we were interested to see if GSK205 could function in animal models in a short term regimen. Wild-type C57/B6J mice were put on high fat diet for 14-15 weeks to induce obesity and insulin resistance. We dosed HFD mice with either GSK205 (10mg/kg) or vehicle twice a day for 7 days. The compound was well tolerated over 7 days, as there was no obvious weight loss in the treated or vehicle control group (Figure 3-7 B). Interestingly, compared to the vehicle treated group, GSK205 treated mice showed significantly reduced mRNA expression of chemokines that have been shown to be regulated by TRPV4, such as Mip1a, Rantes and Vcam (Figure 3-7 C). Consistent with the decrease in these chemokines, mRNA expression of Tnfa was also significantly down-regulated in the treated mice. Other chemokines, including Mcp1 and Mcp3, together with the macrophage marker F4/80, showed a trend towards a decrease but did not reach statistical significance (Figure 3-7 C).

The reduction of the macrophage marker F4/80 and Tnfa indicated that there was less inflammation in the GSK205 treated adipose tissue. We were interested
whether this change would have meaningful effect on systemic insulin resistance and metabolism. We performed intraperitoneal glucose tolerance tests in GSK205 or vehicle treated mice. Consistent with the changes in inflammation, GSK205 treated mice showed significantly improved glucose tolerance (Figure 3-7 D).
Discussion

By an unbiased microarray approach, we unexpectedly discovered that TRPV4 potently regulated pro-inflammatory gene programs in adipocytes. The regulation is beyond the scope of affecting one or two genes, but broadly and profoundly controls the whole set of pro-inflammatory chemotactic genes. The positive regulation of those genes by TRPV4 seems to have a tonic, constitutive component, as the knockdown of the channel potently down-regulated the basal pro-inflammatory gene expression. Yet further activation of TRPV4 had additional merging concept of thermogenic “beige” adipocytes within white adipose tissue hints that there may be a place these tw

As a positive regulator of the pro-inflammatory program, TRPV4 deficiency has substantial effects both in vitro and in vivo. In vivo, the effects seemed to be particularly apparent when animals were developing obesity and insulin resistance. In the context of high fat feeding and obesity, the loss of positive pro-inflammatory TRPV4 signaling is clearly protective. The inflammation is largely attenuated in mice lacking TRPV4. At the same time, the associated insulin sensitivity was partially restored, independently of differences in adiposity.

The most interesting observation is the preliminary therapeutic effects of the TRPV4 antagonist, GSK205. Unlike the genetic knockout model in which TRPV4 was absent chronically and prior to the development of obesity, wild type subjects treated with GSK205 were first on high-fat diet for 14-15 week, by which time the obesity and insulin resistance were quite severe. Relatively short term
administration of GSK205 potently reversed the altered chemokine/cytokine expression in this chronic disease model and significantly improved glucose tolerance, an indication that insulin sensitivity in these mice was partially restored, despite the poor pharmacological properties of this drug. It is promising that, with a better developed compound that targets TRPV4, a better therapeutic effect could be achieved for obesity and metabolic diseases.

Adipose inflammation is viewed as a complex interaction between adipocytes and surrounding cells, including macrophages, T-cells, NK cells and the vasculature. Many molecules that regulate pro-inflammatory signals in adipocytes have been shown to regulate the same signals in immune cells, particularly in macrophages, which shared largely overlapping gene regulation with adipocytes. Also, genes found to regulate pro-inflammatory pathways in adipocytes commonly regulate their responses to external stimulation, such as TNFα or other cytokines, rather than affecting basal gene expression. Therefore, the discovery of TRPV4’s role in controlling a basal, un-stimulated pro-inflammatory gene program in a clonal adipocyte cell line is unusual and striking. The specificity of this regulation to adipocytes and the strong effects on basal gene expression, may suggest that the signaling from TRPV4 could be one of the very early alterations in an adipocyte in response to obesity that then initiates the recruitment of macrophages. More careful analysis throughout the time course of development of obesity in WT and Trpv4-/− mice will be required to further assess this hypothesis.
Materials and Methods

Microarray Analysis

Total RNA was isolated from indicated cell lines and used for microarray analysis. Array hybridization and scanning were performed by the Dana-Farber Cancer Institute Core Facility using Affymetrix Gene Chip Mouse Genome 430A 2.0 arrays according to established methods. For up-regulated gene list, any gene whose expression was below 40 (Arbitrary Unit) in the experimental group was filtered; for down-regulated gene list, any gene whose expression was below 50 (A.U.) in the control group was filtered.

ELISA

Overnight culture medium from fully differentiated adipocytes was used to measure chemokine concentrations. Millplex MAP Mouse Cytokine/Chemokine Panel (Millipore) was used according to manufacturer’s instruction for multiplex detection for MCP1, MIP1α, RANTES, MCP3 and TNFα.

IP-Glucose Tolerance Test

For glucose tolerance tests, animals were fasted overnight. The next morning, glucose levels in tail blood were measured with a standard glucometer prior to and at timed intervals following an intraperitoneal injection of 1.5 g/kg D-glucose. Blood glucose was measured 20, 40, 60, 90 and 120 minute after the glucose injection.

Macrophage isolation and in vitro differentiation
Peritoneal macrophages were isolated three days after thioglycollate challenge and cultured in RPMI, 10%FBS medium. For bone marrow (BM) derived-macrophages, marrow was flushed from the femur and tibia, purified through Ficoll-Paque gradient (Amersham Biosciences), and cultured in DMEM containing 20% FBS and 30% L929 condition medium for 6 days. Differentiated macrophages were counted and re-plated in RPMI medium with 10% FBS for various experiments. For LPS stimulation, cells were pre-treated with IFN-γ (10ng/ml) overnight and exposed to 100ug/ml LSP for 3 hours. For free fatty acid stimulation, cells were treated with 300uM palmitate for 3 hours.
Chapter 4:

Signal Transduction from TRPV4 to Transcription Regulation
**Introduction**

Based on pharmacological and genetic evidence, we have demonstrated that the presence/activation of TRPV4 significantly affects the expression of genes that are important for oxidative metabolism, thermogenesis, and pro-inflammatory pathways in adipocytes. This regulation was identified at the gene expression level, and later we also showed that expression changes caused by TRPV4 indeed have profound effects on cellular and whole-organism physiology, especially in Trpv4-/- mice challenged with a high fat diet.

However, aside from knowing that TRPV4 is a non-selective ion channel that can be activated by a range of chemical and physical stimuli, the mechanism by which TRPV4 controls downstream gene regulation is still unclear. Ion channels have been intensively studied in excitable cells such as neurons or muscle cells, in which ion influx and the associated current is usually the direct functional readout of the channel activation. The lack of literature on TRPV signal-transduction in non-excitable cells, for example, adipocytes, makes it difficult to identify the exact signal transduction cascades from the plasma membrane to any canonical transcriptional regulation.

There are several signaling pathways known to control Pgc1a expression. TRPV4 is a calcium permeable channel and we have shown in a previous chapter that the activation of TRPV4 indeed leads to a rapid increase in total intracellular calcium. Calcium has powerful and broad signaling activity. It has been previous shown that in muscle cells, intracellular calcium can increase
Pgc1a expression, mainly through the activation of MAPK kinase p38 (Lin et al., 2002). However, calcium is a positive regulator of Pgc1a in many cell types (Rohas et al., 2007). This model directly contradicts our finding that TRPV4, a calcium permeable channel, functions as a negative regulator of Pgc1a expression.

cAMP and PKA are also known to regulate Pgc1a and particularly thermogenic gene expression. However, there are no reports suggesting that activation of TRPV4 in other cellular systems would lead to changes in cAMP level or PKA activation. Moreover, cAMP/PKA signaling is very important for activation of the thermogenic program in adipocytes, downstream of beta-adrenergic agonism. However, this is usually a very rapid and acute response. The observation that either antagonist inhibition or shRNA mediated knockdown took more than 24 hours to have any effect in adipocytes, suggests that a slower and multi-step mechanism may be involved.

On the other hand, the signaling pathways that control pro-inflammatory program have been intensively characterized in adipocytes. Stress-activated kinases have been shown to play very important roles in all stages of adipose inflammation and are believed to be a direct cause of insulin resistance by phosphorylating (therefore inhibiting) critical components in the insulin signaling cascades (Rudich et al., 2007). The pro-inflammatory signals they transduce also converge at NF-kB, which then translocate into nucleus to carry out the transcriptional regulation of inflammatory gene expression. Interestingly, in other cells types, it has been reported previously that MAP kinase ERK1/2 can be activated by
TRPV4 activation (Li et al., 2009; Li et al., 2011; Thodeti et al., 2009). This initial observation led us to investigate the role of MAP kinases in mediating signal transduction from TRPV4 to transcriptional control of the downstream genes.
Results

TRPV4 Activation in Adipocytes Leads to Phosphorylation of ERK1/2 and JNK1/2

It has been reported previously that the protein kinases ERK1/2 can be activated by TRPV4 signaling (Li et al., 2009; Li et al., 2011; Thodeti et al., 2009). We therefore examined TRPV4 agonism and activation of three MAP kinases that have been implicated in adipose biology: ERK1/2, JNK1/2 and P38MAPK. Addition of the TRPV4 agonist to 3T3-F442A adipocytes caused a rapid phosphorylation of ERK1/2 at sites known to reflect activation of this kinase (Figure 4-1). The activation appeared as soon as 15 minutes after the addition of the agonist. In contrast, no activating phosphorylation on P38 MAPK was detected with TRPV4 agonism. The β3-agonist CL316243 led to the expected P38MAPK activation in these cells, which was used as a positive control here (Cao et al., 2001) (Figure 4-1). In particular, the activation of ERK1/2 appeared to be dependent on TRPV4, as both basal and stimulated ERK1/2 phosphorylation was largely attenuated by the shRNA against TRPV4. Notably, 2 hours after agonist treatment, ERK phosphorylation in TRPV4 knockdown cells fully returned to baseline, whereas activation of ERK in control cells was sustained for at least 24 hours.

The addition of TRPV4 agonist also induced the potent activation of JNK1/2 as soon as 15 minutes, in control cells. However, the same activation is seen in TRPV4 knockdown cells, and lasted much longer in these cells than
Figure 4-1. TRPV4 agonism leads to the activation of ERK1/2 and JNK1/2. 3T3-F442A adipocytes with shTRPV4 or shGFP were treated with 100nM GSK1016790A for the indicated times, and cell lysates were analyzed by western blot with antibodies against phosphorylated ERK1/2(pERK1/2), JNK1/2(pJNK) and p38 (pP38) or total ERK1/2, JNK1/2 and p38. 20-min treatment of 10uM CL316243 was used as a positive control for p38 phosphorylation.
it did in control cells, suggesting that activation of JNK1/2 was likely not dependent on the presence of TRPV4.

**ERK1/2 Activation Primarily Mediates the Signal from TRPV4 Agonism to Gene Expression**

Inhibitors of MEK1/2 (U0126) and JNK (SP600125) were then used to determine if the activation of these two MAP kinases was required for the key TRPV4-mediated gene regulation events. As shown in Figure 4-2 A, pretreatment of cells with U0126 and SP600125 blocked the TRPV4 agonist-induced phosphorylation of ERK1/2 and JNK1/2, respectively. Interestingly, U0126 effectively reversed the repression on \(Pgc1\alpha\) caused by the agonist (Figure 4-2 B). In contrast, SP600125 had only a small effect.

Concordantly, the induction of \(Mip1\alpha\) and \(Cxcl1\) by the TRPV4 agonist was totally abolished by pre-treating adipocytes with U0126. Pre-treating cells with SP600125 had no effect (Figure 4-2 B). These data strongly suggest that the ERK1/2 protein kinases mediate much of the effect of TRPV4 activation on both the repression of \(Pgc1\alpha\) expression and the induction of many chemokines/cytokines in adipocytes.

**Calcium Influx is Required for TRPV4 Agonism to Activate ERK1/2**

We have demonstrated that the activation of ERK1/2 is required for TRPV4 to regulate both oxidative and pro-inflammatory gene expression. We were interested in the connection between TRPV4 agonism and ERK phosphorylation.
Figure 4-2. ERK1/2 mediates the signal transduction from TRPV4 to gene expression. (A) 3T3-F442A adipocytes were exposed to 100nM GSK1016790A for 15 minutes, with 45-minute pre-treatment of vehicle (GSK101+V), U0126 (GSK101+U) or SP600125 (GSK101+SP), then cell lysates were analyzed by western blot. (B) mRNA expression of Pgc1α, Mip1α and Cxcl1 in these adipocytes were analyzed 48 hours after the treatment. (C) 3T3-F442A adipocytes with control shGFP or shTRPV4 were exposed to 100nM GSK1016790A or 50ng/ml TNFα for 15 minute, in regular DMEM or calcium-free DMEM. Cell lysates were analyzed by western blot. Data are presented as mean ± sem. Student’s t-test was used for single comparisons. * P<0.05, ** P<0.01, *** P<0.001, n.s. not significant, compared to control group.
Because TRPV4 is a calcium permeable channel and the agonist we used here indeed causes a rise in intracellular calcium level, we wanted to understand if the calcium influx, mediated by TRPV4, is causing the activation of ERK1/2.

Both control (shGFP) or TRPV4 knockdown 3T3-F442A adipocytes were exposed to 100nM TRPV4 agonist, in regular DMEM medium or in calcium-free DMEM. While the agonist treatment led to a strong phosphorylation of ERK1/2 within 15 minute in regular DMEM, this activation was absent in adipocytes in the calcium-free medium (Figure 4-2 C). To rule out the possibility that a general defect in kinase activation was caused by calcium deprivation, TNFα, which is known to activate ERK1/2 in adipocyte was used as a positive control for the kinase activity. As shown in Figure 4-2 C, TNFα potently induced ERK1/2 activation regardless of the presence of calcium in the medium. Importantly, in contrast to TNFα, the agonist was unable to activate ERK1/2 in the TRPV4 knockdown cells under either media condition, again confirming that the activation was specific and dependent on TRPV4. Together, these results suggest that calcium influx from medium to cells is specifically required for the activation of ERK1/2 by TRPV4 agonism.
Discussion

TRPV4 is known to be a calcium permeable but non-selective ion channel. Importantly, the mechanisms by which it can affect biological functions are not well understood. As we showed here, transport of calcium into cells is certainly a distinct mechanism, but because TRPV4 is not a calcium specific channel, it is also likely conducting other ions such as Mg or Mn, which may be important for its regulatory function. On the other hand, the presence of a rather large intracellular domain on TRPV4 (Phelps et al., 2010) has also suggested that it could, in fact, also operate as a signal transducing protein. The TRPV proteins are best known as heat sensors and the receptor for capsaicin (TRPV1). It is interesting that TRPV4 has been shown to be activated by cell swelling (Liedtke et al., 2000; Strotmann et al., 2000) and by cellular stretch (Mochizuki et al., 2009; Thodeti et al., 2009). Since fat cells become very large in obesity, it is possible that this cellular distention activates TRPV4 and leads to the expression of the pro-inflammatory gene program as shown here.

The precise mechanisms by which TRPV4 signals are obscure but it is clear that ERK activation is very important for the effects seen here on adipocytes. Interestingly, ERK1 has previously been suggested to play roles in both energy homeostasis and adipose inflammation. ERK1/-/- mice have increased energy expenditure and are resistant to diet-induced obesity (Bost et al., 2005). In a separate study, ERK1 deficiency partially rescued leptin-deficient (ob/ob) mice from insulin resistance by decreasing adipose inflammation (Jager et al., 2011).
Several interesting questions remained here. The precise transcriptional components that connect ERK1/2 to the expression of \textit{Pgc1a} and pro-inflammatory genes are still not clear. What are the transcription factors that activate \textit{Pgc1a} transcription and repress pro-inflammatory chemokines? Are those two programs driven by the same set of transcription factors or not? Or is the change in one program secondary to effects from changes in the other?

Clearly, these are all important aspects that we need to understand better. NF-kB may be an interesting candidate downstream of ERK1/2 and potentially regulates both programs. Its role in promoting pro-inflammatory gene expression is well-characterized. Intriguingly, there was report suggesting NF-kB can negatively modulate the expression of \textit{Pgc1a} in muscle (Coll et al., 2006), particular in diabetic condition. Further investigation would be necessary to look into whether activation of TRPV4 could lead to an alteration in NF-kB activity, presumably via the change of ERK1/2 levels.
Materials and Methods

Materials

Antibody sources are as follows: anti-pERK1/2, ERK1/2, pJNK, JNK, pP38, P38 (Cell Signaling). Forskolin, norepinephrine, GSK1016790A, AMG9810, AM251, insulin, dexamethasone, isobutylmethylxanthine and puromycin were from Sigma. U0126 and SP600125, TNFα were from Cell Signaling. Calcium free DMEM was made by adding 2.5uM EGTA into regular DMEM (Cellgro) then adjust the pH to 7.2.
Chapter 5:

Conclusion and Discussion
Conclusion

This thesis identified the TRP channel family member TRPV4, as a novel regulator of oxidative metabolism, thermogenesis and pro-inflammation gene programs in white adipocytes. A QPCR-based high-throughput small molecule screen initially identified a cannabinoid receptor 1 antagonist that induced \textit{Pgc1a} mRNA expression by an off-target effect on TRPV4 activity. We demonstrated that TRPV4 was a potent negative regulator of PGC1α, affecting mitochondrial biogenesis, OXPHOS and thermogenic capacity of white adipocytes \textit{in vitro}.

An unbiased microarray approach unexpectedly revealed that TRPV4 also positively controlled the expression of an array of pro-inflammatory genes, particularly chemokines. Loss and gain of function studies further demonstrated that TRPV4 functions as a central regulator of a broad chemotactic gene program \textit{in vitro}.

Both the thermogenic and pro-inflammatory aspects of TRPV4 function in adipocytes were further investigated \textit{in vivo} using a genetic model of TRPV4 deficiency. With minimal alteration observed in other oxidative tissues such as skeletal muscle and classical brown fat, \textit{Trpv4-/-} mice have higher thermogenic and brown fat characteristic gene expression in their white adipose tissues compared to wild type littermates. When challenged with a high fat diet, \textit{Trpv4-/-} mice have significantly higher energy expenditure than wild type controls without changes in food intake or movement, and were protected from diet induced obesity. These changes were consistent with the hypothesis that the altered
gene expression in white adipose tissues of Trpv4 -/- mice caused a shift in energy balance by increasing uncoupled respiration-mediated thermogenesis in these tissues.

On the other hand, the up-regulation of a broad pro-inflammatory gene program in white adipose tissue caused by high fat diet was largely attenuated in the Trpv4 -/- mice. Importantly, this reduction seen in TRPV4-deficient mice appeared to be independent of the difference in obesity, which was also a result of TRPV4 deficiency. Consistent with the difference in pro-inflammatory gene expression, Trpv4 -/- adipose tissues have less macrophage infiltration and reduced inflammatory cytokine expression. Trpv4 -/- mice have improved glucose tolerance on high fat diet, suggesting they might be more insulin sensitive as a result of this attenuated inflammation.

Finally, pharmacological manipulation of TRPV4 was preliminarily tested in vitro and in vivo. Gene expression changes in cultured adipocytes as well as in adipose tissue demonstrated that TRPV4 inhibition by drug treatment largely recapitulated the genetic loss of function of Trpv4. Consistent with this gene expression change, the TRPV4 antagonist improved diet-induced insulin resistance in animals.

**Connection between Adipose Thermogenesis and Inflammation**

Adipose cells play a number of key roles in systemic energy balance and metabolic regulation. First, white adipose cells are the primary depot for energy storage in mammals. This important function is highlighted in the tissue steatosis
and illnesses that occurs in individuals with lipodystrophy, a set of syndromes characterized by a localized or generalized deficiency in fat cells. Second, in the context of obesity, where energy intake chronically outstrips energy expenditure, adipose cells become enlarged and adipose tissue becomes inflamed. This was first recognized as a greatly increased expression of TNFα and other cytokines in rodent models of obesity (Hotamisligil et al., 1993). While it was originally believed that fat cells themselves made these cytokines, it is now appreciated that most of the secretion of these molecules comes from immune cells, especially macrophages, that infiltrate adipose tissue in elevated numbers in obesity (Weisberg et al., 2003; Xu et al., 2003). Hence, a critical question is what are the physiological and pathological signals secreted by fat cells that regulate the infiltration and function of these immune cells. Finally, brown adipose cells are an important component of whole body energy homeostasis through the dissipation of stored chemical energy in the form of heat (thermogenesis). The role of brown fat as a defense against both hypothermia and obesity, at least in rodents, is now well established (Feldmann et al., 2009; Lowell et al., 1993). Adult humans have significant depots of brown fat but the contribution made by these deposits to total energy metabolism in man is not known.

Thermogenesis and inflammation are ordinarily considered as two separate aspects of adipose biology. Although they are both important for obesity and metabolic diseases, there has been little data suggesting a regulatory linkage between the two programs in adipocytes. This is probably because, traditionally, thermogenesis was only thought to take place in the classic interscapular brown
fat, which is an anatomically separated organ from most white adipose tissues. Recently, the emerging concept of thermogenic “beige” adipocytes within white adipose tissue hints that there may be a place these two important functions could converge (Ishibashi and Seale, 2010). This was also suggested by the early observation that obesity and HFD led to decreased expression of the β3-adrenergic receptor and impaired oxidative capacity in white adipose tissue (Fromme and Klingenspor, 2011; Lowell and Flier, 1997). Conversely, synthetic PPARγ ligand TZDs have both thermogenic and anti-inflammatory effects in adipose tissue, indicating these two pathways could be coordinately regulated (Petrovic et al., 2010). Interestingly, we showed that TRPV4 was a common cell-autonomous mediator for both thermogenic and pro-inflammatory programs in adipocytes, making TRPV4 the first genetic connection between these two important aspects of adipose biology.

The Endogenous Activation of TRPV4

TRPV4 is known to be a calcium permeable but non-selective ion channel. Importantly, the mechanisms by which it can affect biological functions are not well understood. Several questions remain: what is the biological function of TRPV4 in normal adipocytes? Or, what is TRPV4 “sensing” in adipocytes? Our data suggested TRPV4 was activated in adipocytes during the development of obesity. How does obesity change the activity of TRPV4? It has been suggested that TRPV4 was activated by warm temperature and tonically active at 37°C (Guler et al., 2002; Watanabe et al., 2002). This is consistent with our data that the absence of TRPV4 had profound effects on basal gene expression in
adipocytes. However, our data also suggested TRPV4 activity could be furthered enhanced during the course of HFD; this can hardly be explained by the temperature alone. One explanation would be that certain metabolites associated with obesity, for example, lipid derivatives including endocannabinoids, might affect TRPV4 activity.

It is interesting that TRPV4 has been shown to be activated by cell swelling (Liedtke et al., 2000; Strotmann et al., 2000) and by cellular stretch (Mochizuki et al., 2009; Thodeti et al., 2009). Based on this, it is interesting to hypothesize that the adipocyte hypertrophy might mechanically activate TRPV4. Adipose tissue expansion involves increases in both adipocyte number and cell size. The latter prevails in obesity and is associated with unfavorable metabolic consequences: large adipocytes usually have elevated pro-inflammatory adipokine production and are associated with more macrophage infiltration (Jernas et al., 2006; Murano et al., 2008; Skurk et al., 2007). It has also been noticed that smaller adipocytes are usually associated with higher thermogenic capacity and UCP1 expression (Ghorbani and Himms-Hagen, 1997). Together, hypertrophy is associated with repressed thermogenesis but elevated pro-inflammatory gene expression; both could be resulted from TRPV4 activation. Currently, the mechanism through which hypertrophy alters adipocyte function is unknown, although it has been suggested that the increase of MCP1 expression in large adipocytes involved mechanical stress (Ito et al., 2007). Interestingly, it has been known that TRPV4 can be activated by mechanical stretch in other cells (Thodeti et al., 2009), followed by ERK1/2 activation as we observed in adipocytes after
TRPV4 agonism. Taken together, it is possible that activation of TRPV4 by cell membrane stretch may function as a cell size sensor that controls the downstream metabolic responses to obesity.

In this regard, TRPV4 activation may provide a missing link between obesity and the initiation of “metainflammation” in adipose tissue. In positive energy balance, adipocytes first grow larger to accommodate the demand for lipid storage. When a certain size limit is reached, TRPV4 is activated by the membrane stretch. Similar to the development of atherosclerosis, the initial purpose of this activation might be to recruit macrophages to clear the lipid overflow in adipose tissue. However, if the positive energy balance persists, the pro-inflammatory signals get amplified in a pathological vicious cycle that eventually leads to chronic “metainflammation” in adipose tissue.

**TRPV4 in Other Tissues**

Our data suggests that pharmacologic inhibition of TRPV4 in adipocytes may lead to an increase in energy expenditure and a reduction in adipose tissue inflammation; both could potentially provide therapeutic benefits for obesity and metabolic diseases. Although TRPV4 is expressed at high levels in fat (Liedtke et al., 2000), it is also expressed in many other tissues. Hence the therapeutic value of TRPV4 antagonists in humans may depend on the function of this protein in other tissues (Everaerts et al., 2010). In this regard, it is interesting that a very recent study of *Trpv4* KO mice has also shown a resistance to diet induced obesity, though this paper did not examine adipose tissues in detail (Kusudo et
al., 2011). Instead they showed alterations in muscle biology and fiber-type switching in the soleus muscle. It is not clear how this could affect whole body energy balance and obesity, but the role of TRPV4 in multiple tissues will be important for future studies.

It should also be taken into consideration that TRPV4 might have profound role in bone development and remodeling. It has been reported that the calcium influx mediated by TRPV4 was important for osteoclast differentiation. *Trpv4* ^-/-^ mice developed mild osteopetrosis with aging, likely due to a defect in bone reabsorption (Masuyama et al., 2008). More importantly, it is critical to understand the nature of the TRPV4 mutations that cause skeletal abnormalities in humans before developing strategies to manipulate TRPV4 activity for metabolic diseases.

Other closely related TRPVs, such as TRPV1, may also regulate one or both pathways controlled by TRPV4 in adipocytes. Indeed, our results suggest TRPV1 might have a similar function as TRPV4 in adipocytes, whereas TRPV2 is likely to have an opposite function. Nonetheless, the fact that the genetic ablation of *Trpv4* had a cell-autonomous effect on both the thermogenic and pro-inflammatory programs in adipocytes *in vivo* makes TRPV4 a very promising pharmaceutical target for treating obesity and type 2 diabetes.
References


