TRPV4 Is a Regulator of Adipose Oxidative Metabolism, Inflammation, and Energy Homeostasis

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SUMMARY

PGC1α is a key transcriptional coregulator of oxidative metabolism and thermogenesis. Through a high-throughput chemical screen, we found that molecules antagonizing the TRPVs (transient receptor potential vanilloid), a family of ion channels, induced PGC1α expression in adipocytes. In particular, TRPV4 negatively regulated the expression of PGC1α, UCP1, and cellular respiration. Additionally, it potently controlled the expression of multiple proinflammatory genes involved in the development of insulin resistance. Mice with a null mutation for TRPV4 or wild-type mice treated with a TRPV4 antagonist showed elevated thermogenesis in adipose tissues and were protected from diet-induced obesity, adipose inflammation, and insulin resistance. This role of TRPV4 as a cell-autonomous mediator for both the thermogenic and proinflammatory programs in adipocytes could offer a target for treating obesity and related metabolic diseases.

INTRODUCTION

Brown adipose tissue (BAT) is specialized for the efficient dissipation of chemical energy in the form of heat. It does this by having an exceptionally high mitochondrial content and respiration that is uncoupled from ATP synthesis. This uncoupling is mainly due to the presence of UCP1, a protein that catalyzes proton leak across the inner mitochondrial membrane. Brown fat is very prominent in rodents and human infants, but the presence of substantial brown fat deposits in adult humans has only recently been appreciated (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009).

It is now known that there are two distinct kinds of brown adipocytes. The classical type is exemplified by the interscapular depot of rodents; these UCP1-expressing cells are derived from a muscle-like lineage that expressed *Myf5/Pax7* during earlier development (Lepper and Fan, 2010; Seale et al., 2008). UCP1-positive cells can also emerge in many 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). These cells do not come from a myf5-positive lineage (Seale et al., 2008) and have been called beige or brite fat cells (Ishibashi and Seale, 2010; Petrovic et al., 2010). The regulation of UCP1 and the broader thermogenic gene program in both types of brown adipocytes has been studied in detail. Key transcriptional regulators include FOXC2 (Cederberg et al., 2001), C/EBPβ (Karamanlidis et al., 2007), LXR (Korach-André et al., 2011), PGC1α (Puigserver et al., 1998; Uldry et al., 2006), and PRDM16 (Kajimura et al., 2009; Seale et al., 2007, 2008).

PGC1α was originally identified as a coactivator of PPARγ in the control of the UCP1 promoter in brown adipocytes (Puigserver et al., 1998). Subsequent work has illustrated that it binds to and coactivates many transcription factors (Handschin and Spiegelman, 2006). PGC1α plays a key role in mitochondrial biogenesis and oxidative metabolism in many tissues, 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.

The responsiveness of PGC1α gene expression to external stimuli suggested that it might be possible to find chemical compounds that increase PGC1α expression and function. This in turn might be useful for the treatment of a variety of diseases that would benefit from increased PGC1α or from increased
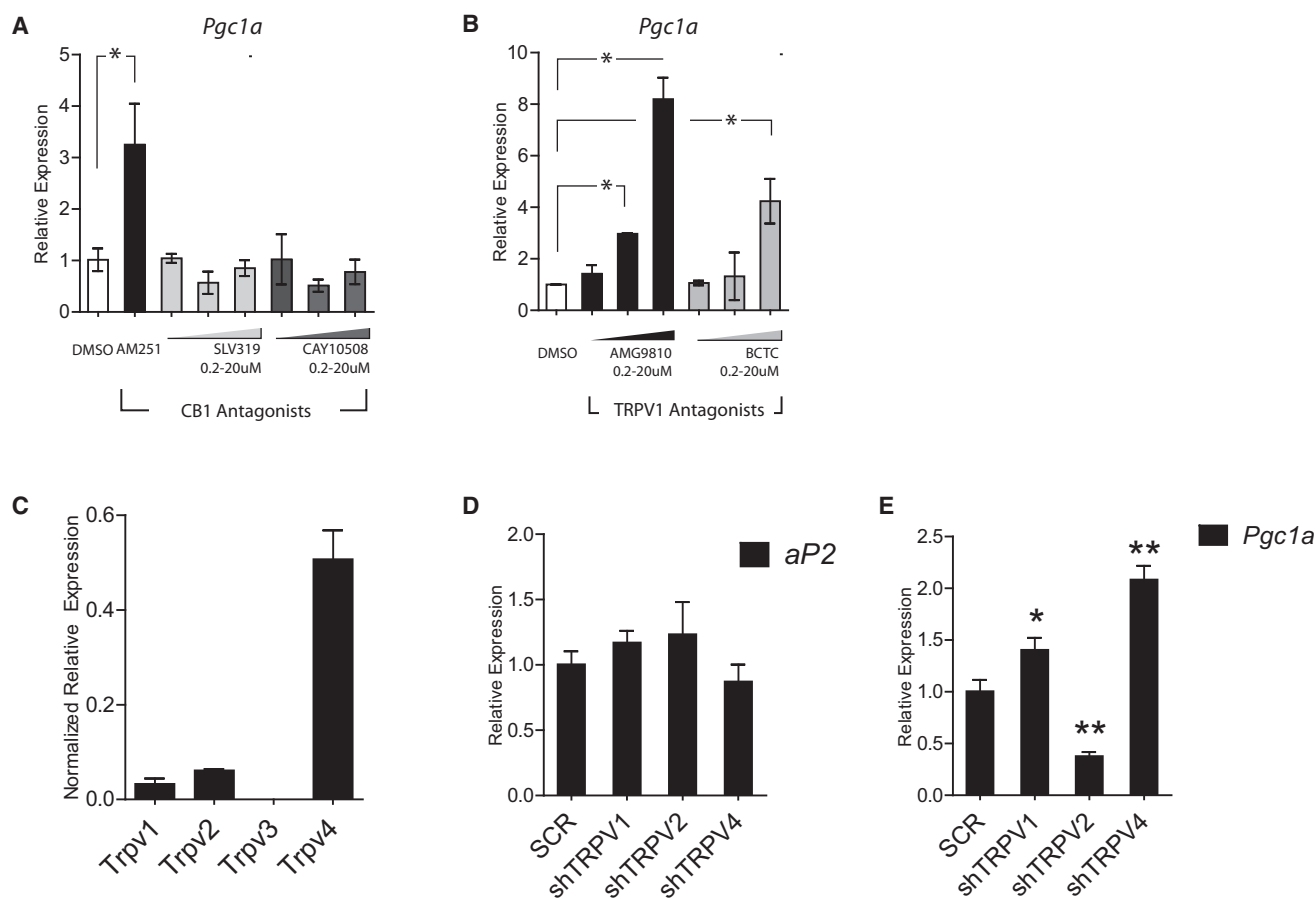
Figure 1. Chemical Screen Identifies TRPVs as Negative Regulators of PGC1α Expression

(A and B) qPCR analysis of *Pgc1α* mRNA in 3T3-F442A adipocytes after 24 hr treatment of CB1 antagonists (A) or TRPV1 antagonists (B). All chemicals were used at 0.2, 2, and 20 μM, except AM251 (20 μM).

(C–E) Normalized mRNA expression of *Trpv1-4* (C). *aP2* (D) and *Pgc1α* (E) mRNA levels in adipocytes infected with scrambled (SCR), shTRPV1, shTRPV2, or shTRPV4 lentivirus.

Data are presented as mean ±SD. See also Figure S1.

mitochondrial activity (Handschin and Spiegelman, 2008). Because elevated PGC1α in muscle plays an antidystrophic and antiatrophic function, we previously screened for drugs and drug-like molecules that elevate PGC1α in murine myotubes (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 white adipocytes. We show here that TRPV4, a member of a family of chemically tractable ion channels, is a negative regulator of PGC1α and the thermogenic gene program. Furthermore, TRPV4 positively regulates a host of proinflammatory genes in

white adipocytes. Genetic ablation and pharmacological inhibition of TRPV4 in mice modulate both thermogenic and proinflammatory pathways in fat, resulting in a robust resistance to obesity and insulin resistance.

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* messenger RNA (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 hr; mRNA from treated cells was then analyzed to quantify the expression of *Pgc1α* (Figure S1 available online). AM-251, a cannabinoid receptor 1 (CB1) antagonist, was identified as one of the primary hits (Figure 1A). AM-251 is a structural analog of a well-known CB1 antagonist, rimonabant (Lan et al., 1999), an antiobesity
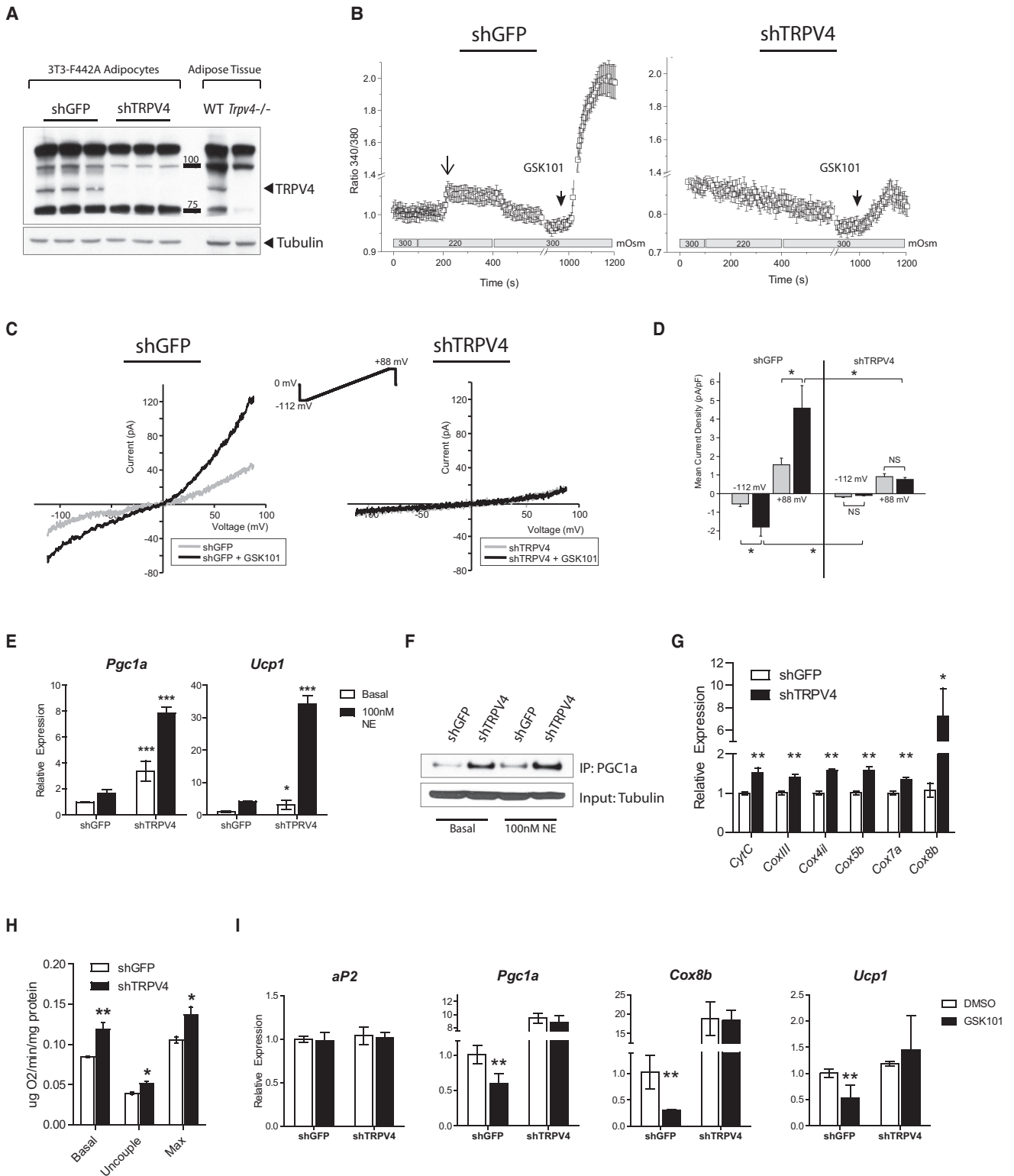
Figure 2. TRPV4 Negatively Regulates Oxidative Metabolism and Respiration in Adipocytes

(A) TRPV4 protein in 3T3-F442A adipocytes with shTRPV4 or shGFP. Adipose tissue lysate from WT and $Trpv4^{-/-}$ mice was used as control.

(B) Intracellular Ca^{2+} rose in response to hypotonicity (220 mOsm, arrow) and to 100 nM GSK1016790A (arrowhead) only in cells with shGFP (n = 48), but not in cells with shTRPV4 (n = 49).

drug that was in clinical use in Europe. Although AM-251 is annotated as a CB1 antagonist, two other CB1 antagonists, SLV319 and CAY10508, failed to induce *Pgc1α* at any dose tested (Figure 1A). Importantly, other molecular targets of AM251 or rimonabant have been reported, including TRPV1 (De Petrocellis et al., 2001; Zygmunt et al., 1999). As shown in Figure 1B, two TRPV1 antagonists, AMG9810 and BCTC, increased *Pgc1α* mRNA expression in adipocytes in a dose-dependent manner (Figure 1B).

AMG9810 antagonizes 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 *Trpv1-4* in 3T3-F442A adipocytes. mRNAs encoding *Trpv1*, *Trpv2*, and *Trpv4* were expressed in 3T3-F442A adipocytes, with *Trpv4* being expressed at the highest level (Figure 1C). To determine which of these channels were regulating *Pgc1α* expression, we used small hairpin RNA (shRNA)-mediated knockdown of each of the expressed TRPVs with lentiviral vectors (Figure S1B). None of the shRNAs appeared to affect adipose differentiation per se, as indicated by the similar expression of the adipose-selective gene *aP2* (Figure 1D). *Pgc1α* mRNA was strongly induced by the shRNA against TRPV4; shRNA against TRPV1 also had a small effect (Figure 1E). These functional data, along with the fact that the expression of *Trpv4* mRNA was ten times higher than that of *Trpv1* in these cells, strongly suggest that TRPV4 was the dominant TPRV family member regulating the induction of *Pgc1α* mRNA by the chemical inhibitors.

TRPV4 Is a Negative Regulator of Oxidative Metabolism and Respiration in Adipocytes

TRPV4 is a calcium-permeable 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, including heat, mechanical stress, anandamide, arachidonic acid, and its derivatives (Everaerts et al., 2010; Nilius et al., 2007). Adipose tissue was shown to have one of the highest levels of *Trpv4* mRNA expression (Liedtke et al., 2000). We found that *Trpv4* expression was higher in white adipose tissues than in brown fat tissue (Figure S2A).

We used retroviral vectors expressing an shRNA against TRPV4 or green fluorescent protein (GFP) to make stable cells with altered TRPV4 expression for biochemical and bioenergetic analyses. Again, the ectopic retroviral shRNA did not appear to affect adipocyte differentiation per se (Figure S2B). We first examined whether there were functional TRPV4 channels present in 3T3-F442A adipocytes. TRPV4 protein was detected by western blot (Figure 2A). In addition, we used Ca^{2+} imaging

to test for TRPV4 activity. Both hypotonicity and the TRPV4 agonist GSK1016790A (Thorneloe et al., 2008; Willette et al., 2008) induced a TRPV4-dependent increase in intracellular calcium in adipocytes (Figure 2B). Consistent with Ca^{2+} imaging results, TRPV4 agonist evoked a TRPV4-like current in control adipocytes, but not in cells expressing an shRNA against TRPV4 (Figures 2C and 2D).

Pgc1α mRNA expression was three times higher in adipocytes expressing shRNA against TRPV4 compared to controls (Figure 2E). Increased PGC1α protein was confirmed by western blot (Figure 2F). β-adrenergic signaling is important for the induction of PGC1α and its thermogenic targets; when cells were exposed to norepinephrine, mRNA expression of *Pgc1α* and *Ucp1* was robustly increased in the TRPV4 knockdown cells compared to controls (Figure 2E). 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*). We observed higher mRNA (Figure 2G) and protein (Figure S2C) expression of these genes in TRPV4 knockdown adipocytes. These changes were dependent on the induction of PGC1α, as the increased expression of these genes was attenuated by expression of an shRNA against PGC1α (Figure S2D).

The increased expression of *Pgc1α*, *Ucp1*, and other mitochondrial genes suggested that TRPV4 inhibition caused the white adipocytes to develop brown-fat-like characteristics, which we termed "browning" here. To determine the physiological impact of this browning gene program, oxygen consumption was measured in adipocytes. As shown in Figure 2H, TRPV4 knockdown adipocytes showed increase in basal, uncoupled, and FCCP-stimulated maximal respiration compared to controls.

We next examined whether chemical activation of TRPV4 would have the opposite impact on these pathways. When added to 3T3-F442A adipocytes, the TRPV4 agonist GSK1016790A repressed the expression of mRNAs encoding *Pgc1α*, *Ucp1*, and *Cox8b* in a TRPV4-dependent manner (Figure 2I). Taken together, these data strongly suggest that TRPV4 functions as a negative regulator of PGC1α and oxidative metabolism in white adipocytes.

TRPV4 Positively Controls a Proinflammatory Gene Program in Adipocytes

To fully understand the function of TRPV4 in adipocytes, microarray analysis was performed with mRNA from 3T3-F442A adipocytes expressing shRNAs against TRPV4 or GFP. As expected, many genes whose expression was strongly increased

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(C) Representative current-voltage plots of endogenous whole-cell Trpv4 current measured in adipocytes with shGFP (right) and shTRPV4 (left) in Tyrode's solution (gray) and upon stimulation with 100 nM GSK1016790A (black). Voltage ramp protocol (500 ms) shown in inset.

(D) Mean current densities at −112mV and +88mV in adipocytes with shGFP (right, n = 6) and shTRPV4 (left, n = 6) in Tyrode's solution (gray) and upon stimulation with 100 nM GSK1016790A (black).

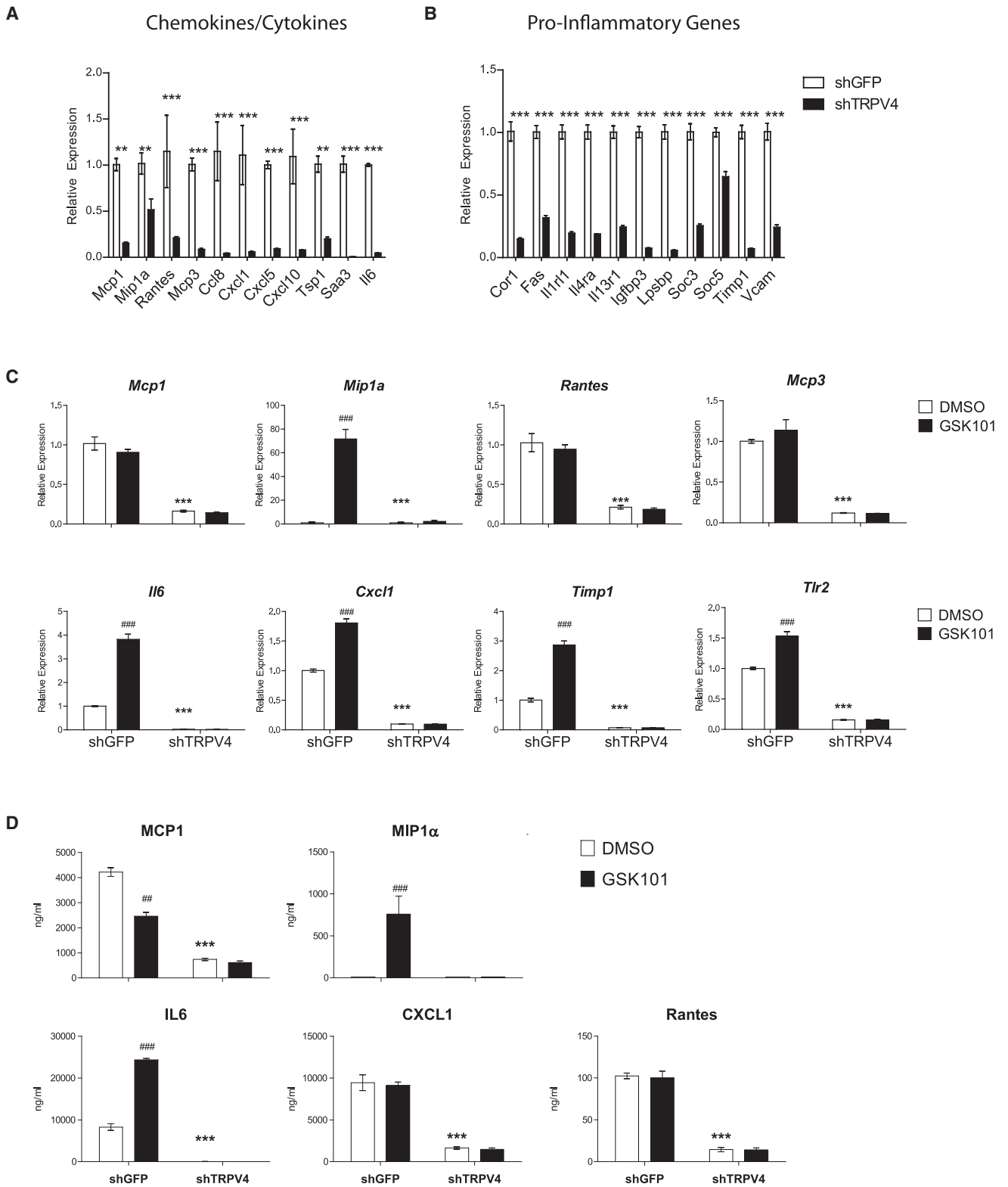(E) *Pgc1α* and *Ucp1* mRNA expression, with or without 100 nM norepinephrine.

(F) PGC1α protein.

(G) mRNA expression of mitochondrial components.

(H) Basal, uncoupled, and FCCP-stimulated oxygen consumption rates.

(I) mRNA expression of *aP2*, *Pgc1a*, *Ucp1*, and *Cox8b* after 48 hr treatment of 100 nM GSK1016790A.

Data are presented as mean ±SD. See also Figure S2.

Figure 3. TRPV4 Controls Proinflammatory Gene Expression in Adipocytes
(A and B) qPCR analysis of mRNA-encoding chemokines/cytokines (A) and genes involved in proinflammatory pathways (B) in 3T3-F442A adipocytes with shTRPV4 or shGFP.

were those involved in brown fat function (Table S1). *Pgc1α* was one of the most highly regulated genes on these arrays.

Strikingly, many genes whose expression was decreased by the TRPV4 knockdown were chemokines/cytokines or genes related to proinflammatory pathways (Table S1). We further analyzed the expression of 22 genes that are either highly regulated by TRPV4 (from the array) or are known to be important in adipose inflammation based on literature. Knockdown of TRPV4 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 3A). 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 3B). Conversely, mRNA expression of *Mip1α*, *Cxcl1*, *Il6*, *Timp1*, and *Tlr2* can be induced by the TRPV4 agonist (Figure 3C). This effect is specific and dependent on TRPV4, as shRNA against TRPV4 fully abolished the induction caused by the agonist (Figure 3C). mRNA expression changes for other adipokines, such as *Adiponectin*, *Leptin*, *RBP4*, and *Resistin*, were also observed (Figure S3).

To determine whether these effects on gene expression resulted in alterations in chemokine secretion, we measured levels of secreted MCP1, MIP1α, CXCL1, IL6, and RANTES in culture medium. Similar to what we observed at the mRNA level, the concentrations of MCP1, MIP1α, CXCL1, IL6, and RANTES were each reduced by more than 85% in the culture medium from the TRPV4 knockdown adipocytes, as compared to controls (Figure 3D). The TRPV4 agonist potently induced MIP1α and IL6 secretion; the secretion of MCP1 was mildly reduced. These data indicate a powerful role for TRPV4 in the regulation of a proinflammatory pathway in adipocytes.

ERK1/2 Mediate Signal Transduction from TRPV4

We investigated the signaling pathways by which TRPV4 carries out these functions in adipocytes. It has been reported that the protein kinases ERK1/2 can be activated by TRPV4 signaling (Li et al., 2009; Thodeti et al., 2009). We therefore examined TRPV4 agonism and the activation of three mitogen-activated protein (MAP) kinases that have been implicated in adipose biology: ERK1/2, JNK1/2, and p38 MAPK. Addition of the TRPV4 agonist to 3T3-F442A adipocytes caused a rapid phosphorylation of both ERK1/2 and JNK1/2 at sites known to reflect activation of these kinases. In contrast, no activating phosphorylation on p38 MAPK was detected with TRPV4 agonism, whereas the β3-agonist CL316243 led to the expected p38 MAPK activation (Cao et al., 2001) (Figure 4A). The activation of ERK1/2 appeared to be dependent on TRPV4 and the presence of extracellular calcium. The phosphorylations caused by the TRPV4 agonist were largely attenuated by the shRNA against TRPV4 or by the depletion of extracellular calcium; in contrast, the phosphorylations caused by TNFα were not affected (Figure 4B).

Inhibitors of MEK1/2 (U0126) and JNK (SP600125) were used to determine whether the activation of these two MAP kinases was required for the key TRPV4-mediated gene regulation events. Pretreatment of cells with U0126 and SP600125 blocked the TRPV4 agonist-induced phosphorylation of ERK1/2 and JNK1/2, respectively (Figure 4C). Interestingly, U0126 effectively reversed the repression on *Pgc1α* caused by the agonist (Figure 4D). In contrast, SP600125 had only a small effect. Concordantly, the induction of *Mip1α* and *Cxcl1* by the TRPV4 agonist was abolished by U0126; SP600125 had no effect. These data strongly suggest that the ERK1/2 protein kinases mediate much of the effect of TRPV4 activation on both the repression of *Pgc1α* expression and the induction of many chemokines/cytokines in adipocytes.

TRPV4-Deficient Mice Have Altered Expression of Thermogenic and Proinflammatory Genes in Adipose Tissue in a Cell-Autonomous Manner

To investigate the function of TRPV4 in adipose tissues in vivo, we studied mice with a genetic deletion of *Trpv4*. These mice are grossly similar to wild-type (WT) animals in morphology, behavior, and breeding (Liedtke and Friedman, 2003). On a chow diet, their body weight is indistinguishable from WT littermates (Figure 5A). In light of the effect of TRPV4 on "browning" and proinflammatory programs in white adipocytes, we examined gene expression in white adipose tissues from *Trpv4*$^{-/-}$ and WT mice.

Subcutaneous (SubQ) 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, SubQ fat from *Trpv4*$^{-/-}$ mice expressed 30-fold higher *Ucp1* mRNA and more UCP1 protein compared to controls (Figures 5B and 5D). A trend toward increased *Pgc1a* mRNA (Figure 5B) and protein (Figure 5C) was also detected in *Trpv4*$^{-/-}$ adipose tissues, however, with substantial variability between individual animals. These mice also have elevated mRNA levels for many genes known to be enriched in BAT, such as *Cidea*, *Cox4il*, and *Cox8b* (Figure 5B). In general, epididymal (EPI) fat has a lower thermogenic capacity; nonetheless, mRNA levels for *Adrb3*, *Pgc1β*, *CytC*, *Cox4il*, and *Cox5a* were significantly higher in EPI fat from the *Trpv4*$^{-/-}$ mice compared to controls (Figure S4D).

We also measured the expression of proinflammatory chemokines, which were 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 between the mutants and controls on a chow diet (Figure 5F).

(C) mRNA expression of *Mcp1*, *Mip1α*, *Rantes*, *Mcp3*, *Il6*, *Cxcl1*, *Timp1*, and *Tlr2* with or without 48 hr GSK1016790A treatment (100 nM).

(D) Protein concentrations of MCP1, MIP1α, CXCL1, IL6, and RANTES in culture medium from adipocytes in (C) were determined by ELISA.

Data are presented as mean ±SD. *p < 0.05, **p < 0.01, and ***p < 0.001, comparing adipocytes with shTRPV4 or shGFP; ##p < 0.01 and ###p < 0.001, comparing cells treated with DMSO or GSK1016790A. See also Figure S3.
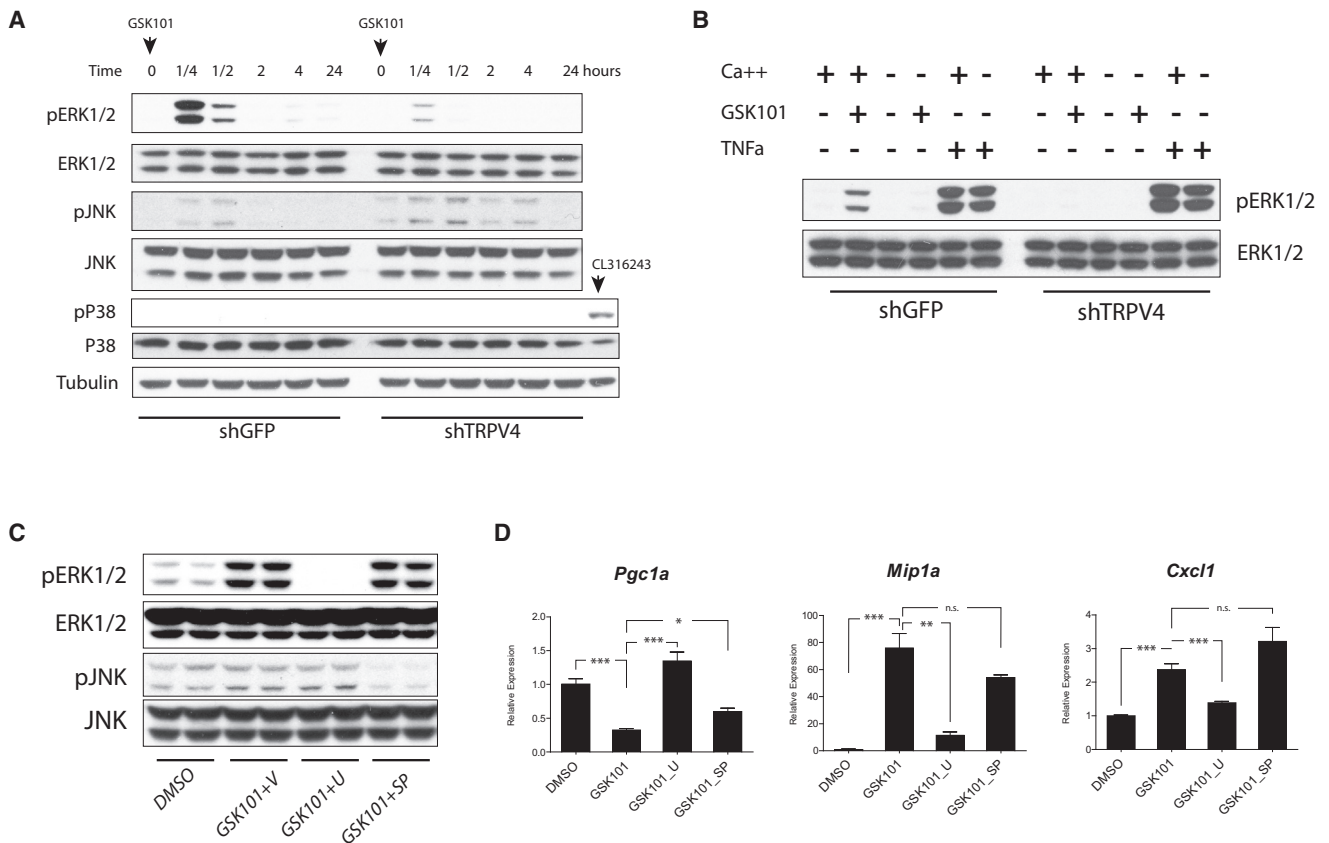
Figure 4. ERK1/2 Mediates the Signal Transduction from TRPV4 to Gene Expression

(A) 3T3-F442A adipocytes with shTRPV4 or shGFP were treated with 100 nM GSK1016790A for the indicated time, and cell lysates were analyzed by western blot. CL316243 (10 μM, 20 min) was used as a positive control for the detection of p38 phosphorylation.

(B) Adipocytes were exposed to 100 nM GSK1016790A or 50 ng/ml TNFα for 15 min in regular or calcium-free DMEM.

(C) Adipocytes were exposed to 100 nM GSK1016790A for 15 min with 45 min pretreatments of vehicle (GSK101+V), 10 μM U0126 (GSK101+U), or 10 μM SP600125 (GSK101+SP).

(D) mRNA expression of *Pgc1α*, *Mip1α*, and *Cxcl1* were analyzed 48 hr after the treatment.

Data are presented as mean ±SD.

To further understand the role of TRPV4 under metabolic stress, we challenged these mice with a high-fat diet (HFD) that would induce obesity and provoke adipose inflammation. There was no significant body weight difference between the *Trpv4*^{−/−} and control mice until the animals were on the HFD for 9 weeks (Figure 5A). Male and female mice showed a similar pattern of weight gain (Figure S4A).

The adipose tissues were first examined after 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, SubQ fat from the *Trpv4*^{−/−} animals expressed higher levels of these genes (Figure S4B). Histological analysis showed that mutant mice have smaller and more UCP1-positive adipocytes compared to controls (Figure 4E).

Eight weeks of the HFD significantly elevated the mRNA expression of proinflammatory chemokines in EPI fat in WT mice, such as *Mcp1*, *Mip1α*, *Rantes*, and *Mcp3*, compared to animals on a chow diet. Interestingly, without a difference in

adiposity at this time point, *Trpv4*^{−/−} mice showed a substantial decrease in the mRNA expression of *Mcp1*, *Mip1α*, and *Mcp3* (Figure 5F). Similarly, the induction of those genes in the SubQ fat was blunted in the *Trpv4*^{−/−} mice (Figure S4E).

As exposure to the HFD extended to 16 weeks, there was no longer a difference in *Ucp1* mRNA between the *Trpv4*^{−/−} and control animals. However, the *Trpv4*^{−/−} mice still had elevated expression of mRNAs encoding *Adrb3* and *Pgc1α* in both the SubQ and EPI depots (Figures S4C and S4D). Chemokine expression in EPI fat continued to rise in WT mice. The expression of mRNAs for *Mip1α* and *Vcam* remained low in the *Trpv4*^{−/−} mice, but the differences in *Mcp1* and *Mcp3* were blunted (Figure 5F).

Because the *Trpv4*^{−/−} mice have a whole-body TRPV4 deficiency, we asked whether the phenotype observed in vivo was associated with cell-autonomous alterations in adipocytes. 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
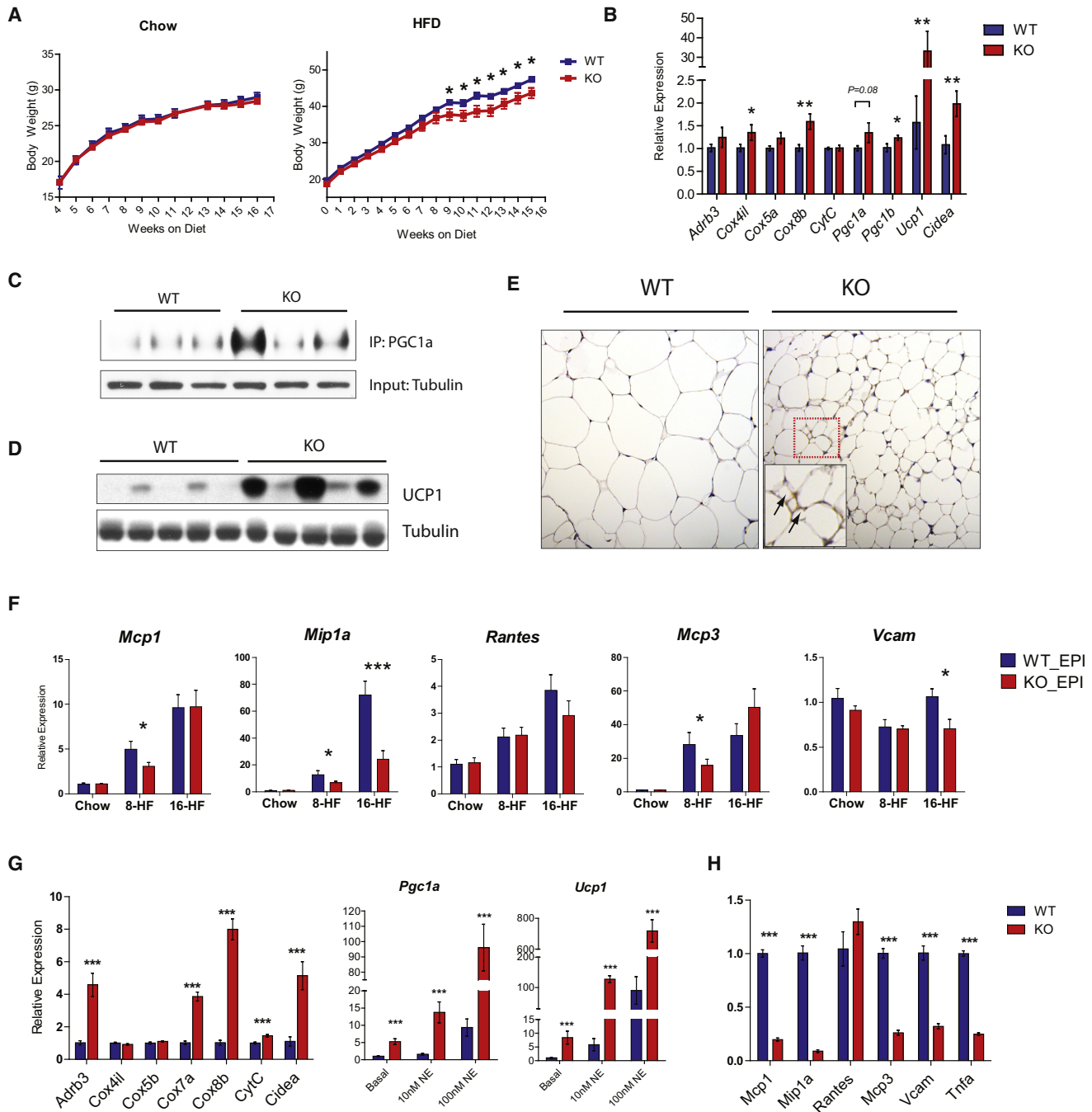
Figure 5. Altered Thermogenic and Proinflammatory Programs in *Trpv4*$^{-/-}$ Adipose Tissue

(A–D) (A) Body weights of WT and *Trpv4*$^{-/-}$ mice on chow and HFD. mRNA expression of thermogenic genes (B), PGC1α protein (C), and UCP1 protein (D) in SubQ fat from chow-fed mice.

(E) Representative images from immunohistochemistry for UCP1 (brown stain) in SubQ fat after 8 weeks of HFD. UCP1-expressing cells are indicated by arrows.

(F) mRNA expression of chemokines in EPI fat was analyzed under three diet conditions.

(G) Thermogenic gene expression and (H) chemokines and *Tnfα* mRNA expression in *Trpv4*$^{-/-}$ and WT primary adipocytes.
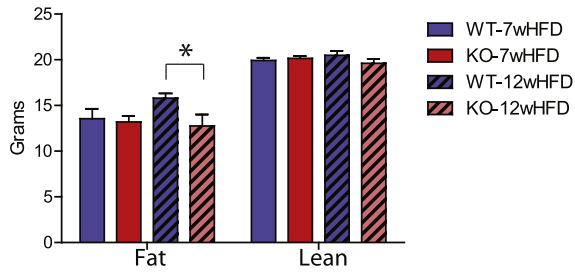
Data are presented as mean ±SEM. See also Figures S4 and S5.

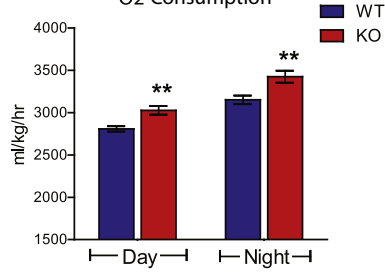than 90% of the cells were fully differentiated. Compared to those from WT controls, adipocytes from *Trpv4*$^{-/-}$ mice showed elevated mRNA expression for "browning" gene program (Figure 5G). Importantly, they had much greater induction in terms of *Pgc1α* and *Ucp1* expression in response to norepinephrine. Conversely, the mRNA expression of proinflammatory genes,
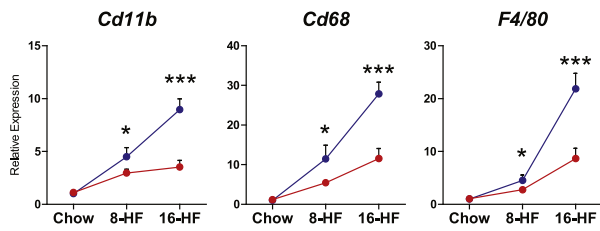
A Body Composition

WT-7wHFD
KO-7wHFD
WT-12wHFD
KO-12wHFD

B O2 Consumption

WT
KO

C

Cd11b *Cd68* *F4/80*

WT
KO

D WT   KO

E

8w-HFD — pSer²⁷³ PPARγ / PPARγ

16w-HFD — pSer²⁷³ PPARγ / PPARγ

WT   KO

F *Tnfa*

WT
KO

G Plasma Insulin

WT
KO

H GTT

p<0.001

I ITT

WT
KO

p<0.001
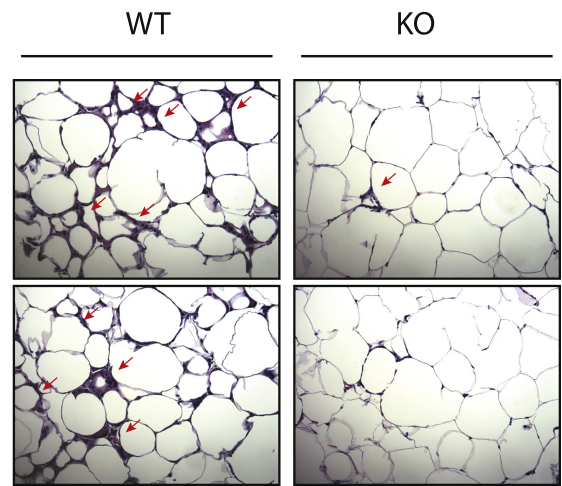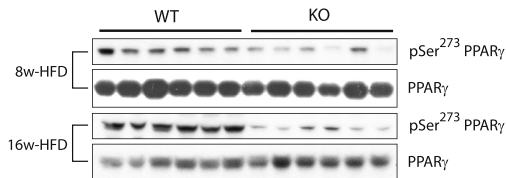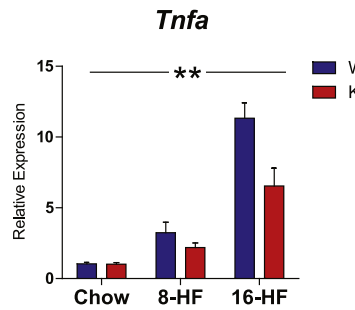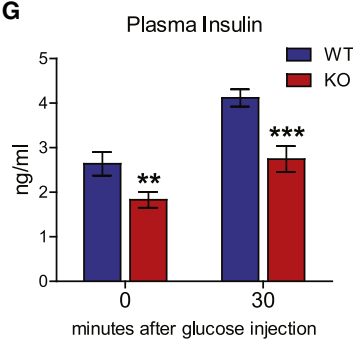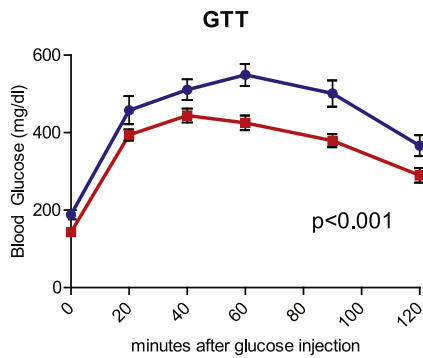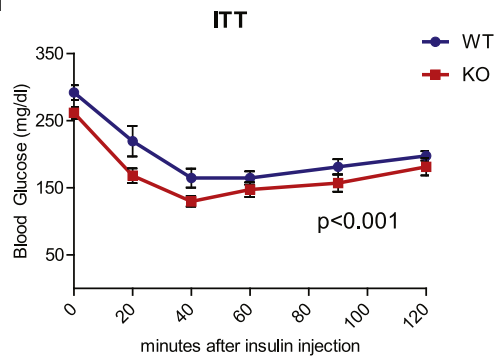
such as *Mcp1*, *Mip1α*, *Mcp3*, *Tnfα*, and *Vcam*, were substantially reduced in *Trpv4*$^{-/-}$ adipocytes (Figure 5H).

Moreover, we analyzed other metabolically active tissues and organs, including interscapular brown fat, skeletal muscle, and liver; minimal changes in expression of genes of oxidative metabolism were observed in these tissues between the WT and *Trpv4*$^{-/-}$ mice (Figures S5A–S5C). Together, these data indicate that TRPV4 controls key gene expression programs in adipocytes in an apparently cell-autonomous manner.

Increased Energy Expenditure Protects TRPV4-Deficient Mice from Diet-Induced Obesity

The TRPV4 mutant mice began to gain significantly less weight after 9 weeks on the HFD compared to WT controls. Body composition analysis showed that *Trpv4*$^{-/-}$ mice had gained less fat mass compared to controls (Figure 6A).

Energy expenditure (EE) in these mice was measured after 7 weeks of HFD, before the body weight of mutants diverged from controls. By normalizing oxygen consumption to body weight (Figure 6B) or by analysis of covariance (ANCOVA) (Tschöp et al., 2012) using body weight and genotype as covariance (p = 0.02 for genotype effect, Table S2), a highly significant increase in EE was observed in the *Trpv4*$^{-/-}$ mice compared to WT controls. Importantly, there was no significant difference in food intake, physical activity, or body temperature between the two groups (Figures S6A–S6C). Together, these data strongly suggest that the reduced weight gain upon HFD in *Trpv4*$^{-/-}$ mice was due, at least in part, to an increased EE associated with an elevated thermogenic gene program in the white adipose tissues.

Trpv4$^{-/-}$ Mice Have Reduced Inflammation in Adipose Tissue and Improved Insulin Sensitivity

Obesity is associated with chronic "metainflammation" in adipose tissue (Hotamisligil, 2006). Although the causes of the recruitment of immune cells have not been well understood, elevated chemokine expression has been suggested to be critical for the development of inflammation and insulin resistance (Sell and Eckel, 2009).

To understand the impact of the changes in chemokine gene expression caused by TRPV4 deficiency, we analyzed the expression of macrophage markers (F4/80, CD68, and CD11b) to quantify the macrophage infiltration in *Trpv4*$^{-/-}$ and WT adipose tissues from all three diet groups. As expected, HFD increased the expression of macrophage markers in WT adipose tissue (Figure 6C), indicating that macrophages were actively recruited into adipose tissues. Consistent with the reduction in chemokine expression, *Trpv4*$^{-/-}$ adipose tissue showed a 40 or 60% reduction in the expression of the macrophage marker after 8 or 16 weeks of HFD, respectively (Figure 6C). Indeed, histologic analysis also showed that there were far fewer "crown-like structures"—which were previously shown to represent macrophages in fat tissues (Cinti et al., 2005)—in the *Trpv4*$^{-/-}$ adipose tissues (Figure 6D). Importantly, macrophages from *Trpv4*$^{-/-}$ mice did not have altered program of gene expression either at the basal level or in response to lipopolysaccharides (LPS) or free fatty acid (Figures S6D and S6E); this strongly suggests that the difference in macrophage infiltration in adipose tissues was likely due to the altered adipocyte function rather than alterations in the macrophages per se.

To further assess the inflammation in adipose tissues, the mRNA expression of *Tnfα*, a key cytokine for obesity-induced insulin resistance, was measured. HFD significantly increased *Tnfα* mRNA in WT adipose tissue, whereas the induction was largely reduced in *Trpv4*$^{-/-}$ mice (Figure 6F). Furthermore, phosphorylation of serine 273 on PPARγ, a modification that is associated with obesity and insulin resistance (Choi et al., 2010), was strongly attenuated in the *Trpv4*$^{-/-}$ adipose tissue compared to WT controls (Figure 6E).

Adipose inflammation is associated with insulin resistance. Consistent with the changes in inflammatory markers, *Trpv4*$^{-/-}$ mice have improved insulin sensitivity, as indicated by the reduced fasting and glucose-stimulated insulin levels (Figure 6G), as well as an improved glucose tolerance (Figure 6H) compared to WT controls. We also observed an improved insulin tolerance (Figure 6I) in the *Trpv4*$^{-/-}$ mice. Importantly, these changes preceded the alterations in body weight (Figures S6F and S6G).

## Pharmacological Inhibition of TRPV4 Modulates an Adipose Gene Program and Improves Glucose Homeostasis In Vivo

The role of TRPV4 in adipose energy metabolism and inflammation makes it a potential target for obesity and insulin resistance. We used the TRPV4 antagonist GSK205 (Phan et al., 2009) to test whether pharmacological modulation of TRPV4 could provide metabolic benefits in a mouse model of obesity and insulin resistance.

GSK205 potently antagonized TRPV4 in 3T3-F442A adipocytes, as it effectively blocked the calcium influx caused by TRPV4 agonist (Figure 7A). Treating these adipocytes with GSK205 for 4 days resulted in increased expression of thermogenic genes (Figure 7B) and was also accompanied by a decrease in the proinflammatory gene program. This shift resembled the gene expression changes seen in TRPV4-deficient adipocytes.

Figure 6. *Trpv4*$^{-/-}$ Mice Are Protected from Obesity, Adipose Inflammation, and Metabolic Dysfunction with Exposure to High-Fat Diet
(A) Body composition in WT and *Trpv4*$^{-/-}$ mice.
(B) Energy expenditure (as oxygen consumption) after 7 weeks of HFD.
(C) mRNA expression of macrophage markers.
(D) Hematoxylin and eosin staining (H&E) of EPI fat after 16 weeks of HFD; arrows indicate "crown-like structures."
(E) Western blot analysis of PPARγ serine-273 phosphorylation in EPI fat.
(F) *Tnfa* mRNA expression in EPI fat.
(G) Fasting and glucose (1 g/kg) stimulated insulin levels.
(H) IP-glucose tolerance test (1.0 g/kg) and (I) IP-insulin tolerance test (1 U/kg) in WT and *Trpv4*$^{-/-}$ mice after 12 weeks of HFD.
Data are presented as mean ±SEM. See also Figure S6.
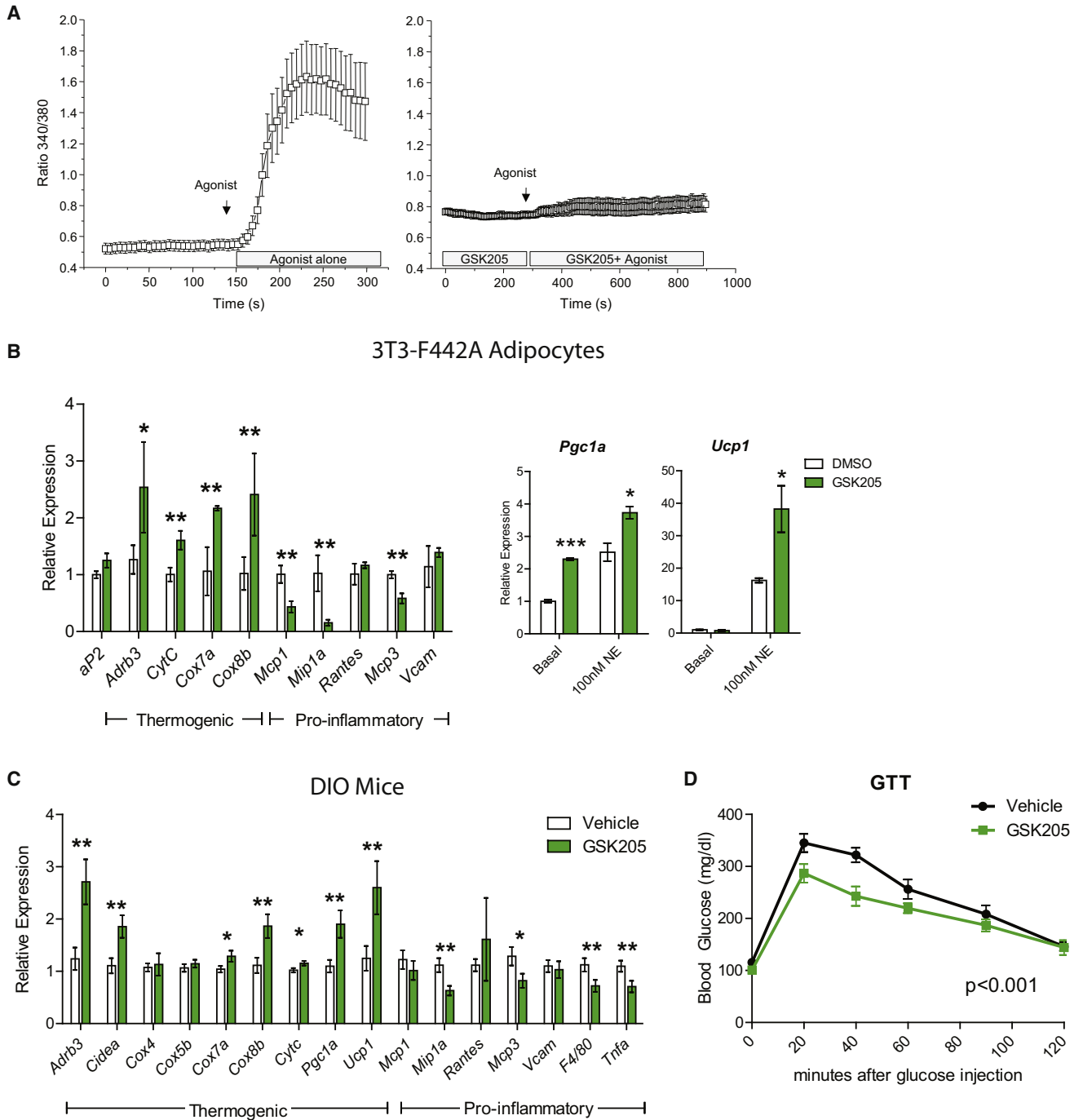
Figure 7. TRPV4 Antagonist Modulates an Adipose Gene Program and Improves Glucose Homeostasis In Vivo

(A) Intracellular Ca^{2+} measurement in 3T3-F442A cells in response to 100 nM GSK101 (agonist, arrowhead), with (n = 13) or without (n = 12) 10 μM TRPV4 antagonist GSK205.

(B and C) (B) The mRNA expression of thermogenic and proinflammatory genes in GSK205- (5 μM) treated 3T3-F442A adipocytes and (C) in EPI fat from GSK205-treated animals.

(D) IP-glucose tolerance test (1 g/kg) in GSK205 or vehicle-treated DIO mice.

Data are presented as mean ±SEM. See also Figure S7.

GSK205 has a relatively short half-life of 2 hr in the plasma and adipose tissues (Figure S7A). As proof of principle, we treated diet-induced obese (DIO) mice with 10 mg/kg GSK205 or vehicle twice daily for a short period (7 days). The compound was relatively well tolerated, as there were no apparent signs of sickness or weight loss in either group during this period (Figure S7B). Compared to controls, GSK205-treated mice showed significantly increased expression of thermogenic genes such as *Ucp1*, *Pgc1a*, *Cidea*, and *Cox8b*. Drug treatment also caused a reduced expression of the proinflammatory chemokines, macrophage marker, and *Tnfa* (Figure 7C) in the EPI fat. These changes largely recapitulated the molecular phenotypes seen in the *Trpv4*$^{-/-}$ mice. A less significant trend in the expression of these genes was observed in the SubQ fat (Figure S7C). In contrast, no significant change was observed in the interscapular BAT (Figure S7D). Consistent with these gene expression changes, strikingly, this short-term GSK205 treatment significantly improved glucose tolerance in DIO mice compared to controls (Figure 7D).

DISCUSSION

Adipocytes play a number of key roles in systemic energy balance and metabolic regulation. First, white adipocytes are the primary depot for energy storage in mammals. This important function is highlighted in the tissue steatosis and illnesses that occur in individuals with lipodystrophy. Second, in the context of obesity, where energy intake chronically outstrips energy expenditure, adipocytes 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). Although 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 now is what are the physiological and pathological signals secreted by fat cells that regulate the infiltration and function of these immune cells? Finally, brown adipocytes are an important component in whole-body energy homeostasis through the dissipation of stored chemical energy in the form of heat. The role of brown fat as a defense against both hypothermia and obesity, at least in rodents, is 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 is not known.

Despite both being important for obesity and related diseases, thermogenesis and inflammation are ordinarily considered as two separate aspects of adipose biology. As a common mediator for both programs, TRPV4 is one of the first examples indicating that these two programs are connected at the molecular and physiological levels. Moreover, the striking changes of chemokine gene expression in an adipocyte cell line (Figure 3A) suggest that signaling from TRPV4 could serve as an early trigger of immune cell chemoattraction. Hence, the convergence of thermogenesis and inflammation identified here not only suggests an angle for targeting obesity but also provides a perspec-tive on understanding the origin of adipose inflammation and insulin resistance.

It is interesting that TRPV4 has been shown to be activated by cellular swelling (Liedtke et al., 2000; Strotmann et al., 2000) and by cellular stretch (Gao et al., 2003; Mochizuki et al., 2009; Thodeti et al., 2009). Because adipocytes become very large in obesity, it is possible that this cellular distention activates TRPV4 and leads to the changes in gene programs. The precise mechanisms by which TRPV4 signals are obscure, but it is clear that ERK1/2 activation is very important for the effects.

TRPV4 deficiency protected mice from diet-induced obesity and insulin resistance. Although the animals studied here have a global *Trpv4* deletion, there are several reasons to believe that white adipose tissues contribute significantly to the phenotypes. First, the gene expression changes in *Trpv4*$^{-/-}$ adipose tissue largely recapitulated what we observed in cultured adipocytes, and many of those changes preceded metabolic intervention and the difference in physiological parameters. In contrast, minimal differences in these key metabolic pathways were observed in other metabolically active organs (liver and muscle) in the same time frame (Figures S5A–S5C). These data strongly suggest that the phenotypes seen in white adipose tissue are unlikely to be secondary to those organs. However, with current data, we certainly cannot exclude potential contributions from the central nervous system (CNS) and the sympathetic nervous system in particular. Also, it is interesting that a very recent study of *Trpv4*$^{-/-}$ mice has also shown a resistance to diet-induced obesity (Kusudo et al., 2012). Although this paper did not examine adipose tissues in detail, they showed alterations in muscle biology and fiber-type switching in the soleus muscle. It is not clear how this could affect energy balance and obesity, but the role of TRPV4 in multiple tissues will be important for future studies.

Our proof-of-principle study suggests that pharmacologic inhibition of TRPV4 leads to an elevation of the thermogenic gene program and a reduction in adipose tissue inflammation—both of which could provide therapeutic benefits for obesity and metabolic diseases. Although TRPV4 is highly expressed in fat, it is also expressed in many other tissues and has been implicated in osmotic regulation (Liedtke and Friedman, 2003), bone formation (Masuyama et al., 2008), and bladder dysfunction (Gevaert et al., 2007). Hence, the size of the therapeutic window of TRPV4 antagonists in metabolic diseases may depend on those functions. In particular, several TRPV4 mutations associated with neurodegenerative disease have been recently identified in humans (Jia et al., 2010; Land-ouré et al., 2010; Phelps et al., 2010). Further characterization on the nature of these mutations would be valuable in determining the therapeutic value of TRPV4 antagonists.

It should be considered that other closely related TRPVs, such as TRPV1, may also regulate one or both pathways controlled by TRPV4 in fat. Several reports have suggested that TRPV1 and TRPM8 could affect adipose function (Ma et al., 2012; Motter and Ahern, 2008; Zhang et al., 2007). Nonetheless, the fact that the genetic ablation and chemical inhibition of TRPV4 has a cell-autonomous effect on both thermogenic and proinflammatory programs in white adipocytes in vivo makes TRPV4 in particular a very promising target for treating obesity and type 2 diabetes.

EXPERIMENTAL PROCEDURES

Materials

Antibody sources are as follows: UCP1, tubulin and OXPHOS (Abcam), TRPV4 (Alomone), p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p38 (Cell Signaling), and PGC1α (Calbiochem). BCTC was from Tocris. SLV319, CAY10508, and rosiglitazone were from Cayman. U0126 and SP600125 were from Cell Signaling. GSK205 was synthesized at the Scripps Research Institute. Other chemicals are from Sigma. shRNA constructs were in pLKO vectors or pMKO vectors. Calcium-free DMEM was made by adding 2.5 mM EGTA into DMEM (Cellgro). Sequences for all shRNA and primers are listed in Table S3.

Chemical Screen

Briefly, after 2 days of differentiation, 3T3-F442A adipocytes were trypsinized and split into 384 well plates (3,000 cell/well). At day 6, cells were treated with the bioactive library (Broad Institute) for 20 hr. mRNA was harvested by using the TurboCapture kit (QIAGEN), which was reverse transcribed to cDNA and quantified by qPCR. All values were normalized to DMSO-treated cells.

Animals

All animal experiments were performed according to procedures approved by the IACUC of Dana-Farber Cancer Institute. Mice were on a standard chow or a 60% high-fat diet (Research Diets) with 12 hr light cycles. $Trpv4^{-/-}$ mice were provided by Dr. Liedtke and backcrossed to C57BL/6J background. Unless specified, male mice were used for experiments. Each group contains 9–16 animals. For drug treatment, C57BL/6J mice were on HFD for 14–15 weeks before treatment. GSK205 (in DMSO) was dissolved in a vehicle contains 5% Tween80 and 90% saline before intraperitoneal injection. Each group contains 10–12 mice, and experiments were performed twice in independent cohorts.

Metabolic Phenotyping

For glucose tolerance tests, animals were fasted overnight. Glucose levels in tail blood were measured with a standard glucometer prior to and at indicated intervals following an intraperitoneal injection of D-glucose. For insulin tolerance tests, animals were fasted for 4 hr before experiments. Fat and lean mass were measured by MRI. Energy expenditure was evaluated by using a Comprehensive Lab Animal Monitoring System (Columbia Instruments). Mice were acclimated in the metabolic chambers for 2 days before the experiments. CO_2 and O_2 levels were collected every 32 min for each mouse during a period of 2 days and were normalized to total body weight. Movement and food intake are measured more frequently at regular intervals.

Cell Culture

For virus production, 293T (for lentivirus) or phoenix cells (retrovirus) were transfected with Fugene 6 (Roche) with viral vectors. Viral supernatant was harvested 48 hr later. 3T3-F442A preadipocytes were infected for 4 hr (lenti) or overnight (retro), followed by puromycin selection (2 μg/ml). 3T3-F442A adipocyte differentiation was induced by treating confluent cells with 850 nM insulin for 8–10 days. To stimulate thermogenesis, cells were incubated with norepinephrine for 4 hr. For primary adipocytes, stromal vascular fractions (SVF) from inguinal fat of 5-week-old male mice were prepared and differentiated for 8 days as previously described (Kajimura et al., 2009).

Statistics

Student's t test was used for single comparisons. Two-way ANOVA (repeated measurement) was used for GTT and ITT. Unless specified, *p < 0.05, **p < 0.01, ***p < 0.001, and not significant (n.s.) p > 0.05.

ACCESSION NUMBERS

The GEO accession number for the microarray data reported in this paper is GSE40280.

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