Celastrol Protects against Obesity and Metabolic Dysfunction through Activation of a HSF1-PGC1α Transcriptional Axis

Graphical Abstract

Highlights
- HSF1 regulates PGC1α-dependent metabolic programs in adipose tissues and muscle
- Celastrol increases mitochondrial function in fat and muscle via HSF1
- Celastrol enhances energy expenditure and protects mice on HFD from developing obesity
- Celastrol increases muscle endurance

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In Brief
Ma et al. identify the cellular thermal sensor HSF1 as an upstream activator of PGC1α. The natural compound celastrol activates HSF1 and protects against obesity and metabolic dysfunction by increasing energy expenditure, inducing iWAT browning, BAT activation, and mitochondrial genes in muscle.

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Article

Celastrol Protects against Obesity and Metabolic Dysfunction through Activation of a HSF1-PGC1α Transcriptional Axis

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SUMMARY

Altering the balance between energy intake and expenditure is a potential strategy for treating obesity and metabolic syndrome. Nonetheless, despite years of progress in identifying diverse molecular targets, biological-based therapies are limited. Here we demonstrate that heat shock factor 1 (HSF1) regulates energy expenditure through activation of a PGC1α-dependent metabolic program in adipose tissues and muscle. Genetic modulation of HSF1 levels altered white fat remodeling and thermogenesis, and pharmacological activation of HSF1 via celastrol was associated with enhanced energy expenditure, increased mitochondrial function in fat and muscle and protection against obesity, insulin resistance, and hepatic steatosis during high-fat diet regimens. The beneficial metabolic changes elicited by celastrol were abrogated in HSF1 knockout mice. Overall, our findings identify the temperature sensor HSF1 as a regulator of energy metabolism and demonstrate that augmenting HSF1 via celastrol represents a possible therapeutic strategy to treat obesity and its myriad metabolic consequences.

INTRODUCTION

The accumulation of excessive fat tissue, the result of an imbalance between energy input and its output, leads to obesity, diabetes, cardiovascular disease, and hepatosteatosis, which together represent the leading causes of adult morbidity and mortality worldwide. The main site of energy storage is white adipose tissue (Farmer, 2008; Mueller, 2014; Gesta et al., 2007), while brown fat is principally involved in energy expenditure via thermogenesis, achieved through the dissipation of chemical energy via the activity of Uncoupling Protein 1 (UCP1), a mitochondrial protein uncoupling respiration from ATP synthesis. For a considerable period of time this rigid framework defined our view of how energy balance was controlled. Recently, this viewpoint has been altered to incorporate the observation that adult humans contain measurable amounts of brown fat (Cypess et al., 2009; Skarulis et al., 2010; Ouellet et al., 2012). Recently a new cell type of smooth muscle origin (Long et al., 2014), called brite or beige, has been shown to be inducible upon cold or hormonal stimuli, causing brown remodeling of white fat, expressing UCP1 and contributing to energy expenditure (Nedergaard and Cannon, 2014; Wu et al., 2012; Sharp et al., 2012). In addition, beige cells directly sense temperature changes, suggesting a cell-autonomous mechanism to activate thermogenesis in brite/beige fat depots (Ye et al., 2013). This newly discovered “plasticity” of adipocytes has intensified efforts to identify novel methods to treat obesity and insulin resistance by increasing browning and peripheral energy expenditure (Boss and Farmer, 2012; Cypess and Kahn, 2010).

Mitochondrial dysfunction has been shown to be associated with the development of obesity and insulin resistance (Bournat and Brown, 2010; Patti and Corvera, 2010). The peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a central transcriptional regulator of mitochondrial and peroxisomal remodeling and biogenesis (Wu et al., 1999; Bagattin et al., 2010). Genetic analysis of PGC1α requirement in vivo via generation of total and conditional knockout mice has revealed its involvement in thermogenesis, mitochondrial gene expression (Lin et al., 2004; Leone et al., 2005), and browning of white fat tissues (Kleiner et al., 2012). In addition to fat, skeletal muscle represents a critical metabolic organ in energy homeostasis. It has been shown that PGC1α controls mitochondrial and oxidative fiber conversion gene programs in muscle to increase energy expenditure to match metabolic needs (Handschin and Spiegelman, 2006; Lagouge et al., 2006). Interestingly, mice with neuronal inactivation of PGC1α show hyperactivity and resistance to diet-induced obesity but normal adaptive response to cold exposure, suggesting critical metabolic roles of PGC1α in both central and peripheral tissues (Ma et al., 2010). PGC1α is tightly regulated by a number of signal transduction effectors,
such as AMPK, SIRT1, mTOR, and GCN5, which orchestrate posttranslational modifications that either enhance or repress PGC1α activity in brown adipose tissue, muscle, and liver (Cantó and Auwerx, 2009; Cunningham et al., 2007; Lerin et al., 2006) and transcriptionally induced by thermal, oxidative, and mechanical challenges (St-Pierre et al., 2006; Handschin et al., 2007; Egan et al., 2010).

In response to stress, activated heat shock factor 1 (HSF1) exerts pleiotropic effects (Anckar and Sistonen, 2011). In addition to the classic induction by heat shock, HSF1 is also activated by cold temperatures (Cullen and Sarge, 1997) and plays a role in circadian changes in body temperature through clock gene regulation (Reinke et al., 2008). To date, there has been no mechanistic link between HSF1 and PGC1α, although a recent genome-wide analysis of PGC1α binding sites in transformed hepatocytes raised the possibility of potential occupancy of PGC1α at heat shock response elements in liver cells (Charos et al., 2012). HSF1’s most explored function to date has been the regulation of the expression of genes encoding for chaperone proteins to protect cells against temperature changes, oxidative stress, and hypoxia. In response to these stimuli, HSF1 is activated via the dissociation of HSP90, HSP70, and HSP40, and, upon multimerization and posttranslational modifications (Xu et al., 2012), HSF1 binds to DNA to induce its downstream gene targets through three types of heat shock elements (HSEs). These elements are defined depending on the organization of their GAA/TTC core domains as classic, gap type, or TTC rich (Eastmond and Nelson, 2006; Guo et al., 2008). HSF1 activity can be modulated by a variety of compounds with different chemical structures (Fujikake et al., 2008). One such agent is celastrol, a compound that is known to be capable of inducing classic HSF1 heat shock target genes (Westerheide et al., 2004). Despite the number of reports on the biological functions of HSF1, no studies have yet specifically evaluated the effects of modulation of HSF1 levels and activity on energy metabolism. Here, we directly linked the activation of HSF1 to the induction of PGC1α gene expression and to that of PGC1α downstream programs, thereby defining a critical regulatory axis between these two transcription factors. We show that activation of HSF1 by the natural compound celastrol has profound effects in vitro and in vivo in mice on high-fat diet (HFD) by increasing their energy expenditure, inducing browning of iWAT and brown fat programs in BAT, and activating mitochondrial gene targets in muscle. Our work identifies HSF1 as an important metabolic regulator in mesenchymal cells and tissues and proposes a potential pathway that can be activated for the treatment of obesity and related metabolic disorders.

RESULTS

HSF1 Regulates PGC1α Levels and Brown and Mitochondrial Gene Programs In Vitro

Given the established role of PGC1α in browning of white fat tissue (Wu et al., 1999, Kleiner et al., 2012) and in increasing mitochondrial function in muscle (Lin et al., 2002), we aimed to identify upstream regulators of PGC1α and used the transcriptional activation of PGC1α as a readout to characterize novel regulators of energy expenditure. By performing an in silico screen of transcription factor binding elements in the regulatory region upstream of the PGC1α start site, we identified multiple repeats exhibiting the characteristic core motif 5’-nTTCn-3’ (see Figure S1A available online), previously described as a noncanonical TTC-rich binding site for the heat shock transcription factor HSF1 (Eastmond and Nelson, 2006; Guo et al., 2008). Electromobility shift analysis and luciferase reporter assays confirmed HSF1’s binding at nucleotides −2,369 to −2,346 in the mouse PGC1α promoter (Figures 1A and S1B). To determine the physiological relevance of this finding, we performed chromatin immunoprecipitation (ChIP) assays and showed increased HSF1 occupancy at this site, but not at control sites, in BAT and iWAT of mice exposed to cold temperatures (Figures 1B and S1C). Given the previously reported role of PGC1α in the potentiation of its own expression (Handschin et al., 2003), we next performed coimmunoprecipitation studies to assess the interaction between PGC1α and HSF1 on the PGC1α promoter. Our analysis revealed that HSF1 binds to PGC1α and that this interaction is enhanced by the addition of the HSF1 activator celastrol (Figure 1C). Furthermore, ChIP and re-ChIP studies confirmed that PGC1α and HSF1 co-occupy selectively the HSE in the PGC1α promoter (Figures 1D, S1D, and S1E).

We next used the mesenchymal cell line 10T1/2, previously shown to induce endogenous PGC1α levels in response to a variety of stimuli (St-Pierre et al., 2006), to test the effects of HSF1 modulation in vitro. Forced HSF1 expression increased PGC1α mRNA levels, which were further elevated after the addition of celastrol (Figure S1F). The ability of HSF1 to induce PGC1α mRNA was also confirmed using additional activators of HSF1 (Fujikake et al., 2008), such as the chemical compounds 17-allylamino-17-demethoxy-geldanamycin (17-AAG), geranylgeranylacetone (GAA), or radicicol (RA) and through classic heat shock stimulation (Figure S1G). These results indicated that PGC1α levels are indeed elevated as a result of HSF1 activation, rather than the consequence of nonspecific, off-target effects of selected compounds. Analysis of isofrom selectivity revealed that PGC1α.1 is the only PGC family member regulated by HSF1 and that there are no compensatory changes in PGC1β (Figure S1H). Further analysis of HSF1’s activation revealed the elevation of brown fat selective markers, such as PRDM16 and UCP1, as well as mitochondrial and fatty acid metabolism genes (Figures 1E and S1I–1L), but no induction of differentiation genes (Figure S1M). The heat shock protein HSP70, an established gene target of HSF1, served as a marker for the efficacy of the activators used to elicit HSF1-mediated transcriptional responses.

Given the presence of a HSE in the upstream regulatory regions of the human PGC1α gene (Figure S1A), we tested the effects of HSF1 activation in cells obtained from subcutaneous fat of obese patients. Similarly to murine cells, brown-specific gene expression programs were increased by celastrol in differentiated stromal-vascular fraction (SVF) cells obtained from obese subjects, thereby confirming the role of HSF1 in the regulation of PGC1α and brown fat and mitochondrial programs in human cells as well (Figure S1N). Furthermore, ablation of HSF1 function via HSF1 siRNA knockdown reduced the expression levels of PGC1α and that of its target genes, further confirming that HSF1 regulates PGC1α and brown fat markers (Figures 1F and S1O). Given that HSF1 binds to the PGC1α promoter after acute cold exposure (Figure 1B) and the previously reported evidence
of fat cells’ intrinsic capability to sense cold temperatures, we assessed the potential involvement of HSF1 in cell-autonomous cold sensing. We exposed control or HSF1 siRNA expressing cells to 31°C, a temperature known to induce the expression of UCP1 in isolated cells (Ye et al., 2013), and demonstrated that while control cells responded to cooling by increasing PGC1α and UCP1 levels, cells with HSF1 knockdown had a blunted induction of these thermogenic factors (Figures S1P–S1R). These data suggest a potential role for HSF1 in fat-cell-autonomous temperature sensing and in eliciting thermogenic responses.

**HSF1 Null Mice Have Impaired Energy Metabolism**

Based on the observation that HSF1 regulates the expression of brown fat markers and mitochondrial genes in vitro (Figure 1), we next performed genetic analyses to determine whether modulation of HSF1 levels in vivo would alter metabolism. Given that no studies to date have assessed the impact of HSF1 on energy balance, we characterized the metabolic consequences of HSF1’s ablation in mice. HSF1 null mice showed increased brown (BAT) and inguinal fat depots (iWAT) (Figure 2A), enhanced lipid deposition (Figure 2B), decreased levels of brown fat markers (Figure 2C), and reduced UCP1 staining and levels in iWAT (Figures 2D and 2E). These alterations were associated with decreased total body oxygen consumption (Figure 2F), impaired cold resistance (Figure 2G), and reduced levels of PGC1α and UCP1 in brown and inguinal fat tissue of cold-exposed HSF1 null mice (Figure 2H), with no concomitant changes in food intake nor in locomotor activity (Figures S2A and S2B). The impaired gene expression and metabolic responses observed in HSF1 null mice were recapitulated in isolated cells obtained from BAT and iWAT, showing reduced PGC1α and UCP1 gene expression (Figure S2C), suggesting a cell-autonomous effect of HSF1’s deletion on energetics. Taken together, these data demonstrate that HSF1’s ablation in mice alters mitochondrial and brown fat gene programs in brown and inguinal fat tissues and affects thermogenic function and energy expenditure.

**HSF1 Regulates White Fat Browning through PGC1α**

Given the importance of inducible brown adipocytes in white fat (Harms and Seale, 2013), we next asked if modulation of HSF1’s levels specifically in white fat depots could recapitulate, at least in part, the effects of HSF1’s deficiency on brown and white fat observed in HSF1 null mice. Several methods have been recently used to study tissue- and depot-selective effects of gain or loss of function of specific genes circumventing the generation of conditional knockout mice (Kraus et al., 2014; Whittle et al., 2012; Qian et al., 2013). We took advantage of adenoviral delivery techniques we previously used to efficiently up- or downregulate genes of interest selectively in inguinal fat tissues of C57BL/6J mice (Ma et al., 2014). HSF1 depletion in iWAT was associated with increased size of subcutaneous fat cells, decreased UCP1 staining, and reduced levels of brown fat markers (Figures 3A–3D). Conversely, increased HSF1 expression in iWAT was associated with induction of browning, brown fat gene programs, and PGC1α and UCP1 protein levels (Figures 3E–3H). The delivery of shHSF1 and HSF1 adenoviruses appeared to be specific to iWAT, given that HSF1 levels were modulated only in this depot (Figures S3A and S3B).
Figure 2. HSF1 Null Mice Have Impaired Energy Metabolism

(A–H) Analysis of 2-month-old WT and HSF1 null (HSF1 KO) mice.
(A) Weights of brown (BAT), inguinal (iWAT), epididymal (eWAT), mesenteric (mWAT), and retroperitoneal (rWAT) fat pads.
(B) Representative H&E staining of BAT and iWAT.
(C) Gene expression analysis of brown fat markers in BAT and iWAT.
(D and E) Representative UCP1 staining and UCP1 protein levels in iWAT.
(F) Oxygen consumption levels. ANCOVA analysis using body weight and genotype as covariance shows significant decrease in oxygen consumption in HSF1 null mice (p = 0.018 at room temperature and 0.046 at thermoneutrality, n = 4).
(G) Core temperatures during cold exposure.
(H) PGC1α and UCP1 levels in BAT and iWAT obtained from mice at room temperature (RT) or after 6 hr of cold exposure (4°C). Annotation indicates significant effect of a, genotype; b, temperature; or c, significant temperature-genotype interaction using two-way ANOVA analysis.
(I) Oxygen consumption rate (OCR) in differentiated primary SVF cells from BAT and iWAT of 2-month-old WT and HSF1 null mice in basal conditions, in the presence of 1 μM oligomycin, 1 μM FCCP, or 0.5 μM Antimycin A. OCR is calculated by subtracting OCR measured after antimycin addition from basal OCR, from OCR after oligomycin addition, or from OCR after FCCP addition.

Data are presented as mean ± SEM and *p < 0.05, **p < 0.01 compared to control group.

n = 6 per group unless stated otherwise.
S3B). Overall these data further support a fat-cell-autonomous function of HSF1.

Given HSF1’s ability to induce PGC1α and its downstream mitochondrial, brown fat, and energy utilization genes in vitro and in vivo (Figures 1–3), we next examined the requirement of PGC1α for HSF1-mediated regulation of these gene programs. As shown in Figures 3I–3K, ectopic expression of HSF1 in iWAT was associated with increased levels of mitochondrial and brown fat gene expression and improved cold tolerance in WT, but not in PGClα KO mice. These results demonstrate that PGClα is necessary for HSF1’s metabolic action in inguinal fat tissues.

Celastrol Elicits Metabolic Effects through HSF1 and PGClα

Having established the existence of the HSF1-PGClα axis, we next tested whether pharmacological activation of this axis...
in vivo would lead to enhanced brown fat functions. We chose to treat mice with the HSF1 activator celastrol, due to the clear in vitro effects of this compound on HSF1 and PGC1α (Figures 1A and 1C–1F), because of celastrol’s known favorable safety profile in mice (Kiaei et al., 2005; Yang et al., 2006; Paris et al., 2010) and since our data suggested it has potent effects on primary human cells (Figure S1N). We first determined the specificity of celastrol’s function and the genetic requirement of HSF1 and PGC1α for the celastrol-mediated activation of metabolic target genes. As shown in Figures 4A and 4B, the induction of thermogenic and mitochondrial gene programs elicited by celastrol in WT cells was markedly blunted in cells derived from either HSF1 or PGC1α null mice. To further determine whether the effects of celastrol were fat-cell-autonomous and dependent in vivo on the HSF1-PGC1α axis, we measured celastrol-mediated activation of metabolic genes in mice with HSF1 or PGC1α depletion selectively in iWAT and showed attenuated induction of mitochondrial, brown fat, and fatty acid utilization genes in response to treatment in the absence of either HSF1 or PGC1α (Figure 4C). Together, these data demonstrate that celastrol activates mitochondrial and thermogenic gene programs specifically through HSF1 and PGC1α.

**HSF1 Activation by Celastrol Increases Energy Expenditure**

Having demonstrated the specificity of the HSF1’s activator celastrol, we next tested whether treatment of mice with this compound would lead to reprogramming of metabolic signatures in fat and other tissues. As shown in Figure 5A, the levels of PGC1α and of genes involved in brown fat and mitochondrial function, thermogenesis and lipid oxidation were increased in brown fat tissues of celastrol-treated mice, with no concomitant increase in adipogenic gene programs (Figure S4A). In addition, transmission electron microscopy (TEM) examination of BAT sampled from celastrol-treated animals showed increased mitochondrial number, as well as better preservation of mitochondrial cristae structure (Figure 5B), and molecular analysis demonstrated induction of classic markers of brown fat cells and beige cells (Figure 5C), increased UCP1 levels, and staining in iWAT (Figures 5D and 5E), with no effects on adipogenic gene programs (Figure S4A). Consistent with these molecular changes, celastrol-treated mice exhibited improved cold tolerance (Figure 5F). A similar induction of thermogenic and mitochondrial gene expression profiles (Figures S4B and S4C) and effects on browning (Figures S4D and S4E) were also observed in mice on chow diet treated with celastrol.
Figure 5. HSF1 Activation by Celastrol Induces Mitochondrial, Brown, and Beige Fat Programs in Adipose Tissues and Increases Energy Expenditure

(A–G) Analysis of mice on HFD, untreated (HFD, n = 6), or treated with celastrol at 1 mg/kg/day (HFD + CEL1, n = 6) or 3 mg/kg/day (HFD + CEL3, n = 7).

(A) Gene expression levels of brown fat programs in BAT.

(B) Representative transmission electron microscopy images (TEM) and quantification of mitochondrial area in BAT.

(C) Gene expression analysis of brown and beige selective genes in iWAT.

(D and E) Representative UCP1 staining and UCP1 protein levels of iWAT.

(F) Core body temperatures during cold exposure.

(G) Oxygen consumption levels in mice treated with or without CEL for 3 days at room temperature (RT) or at thermoneutrality (30°C), n = 6.

(I) Oxygen consumption rate in differentiated 10T1/2 cells expressing control or siHSF1 in the presence of vehicle or 0.5 μM CEL for 72 hr, in basal conditions, or with 0.5 μM oligomycin, 1 μM FCCP, or 0.5 μM antimycin A. ΔOCR is calculated by subtracting OCR measured after antimycin addition from basal OCR, from OCR after oligomycin addition, or from OCR after FCCP addition.

Data are presented as mean ± SEM and *p < 0.05, **p < 0.01 compared to control group.
We next determined the effects of pharmacological activation of HSF1 on energy expenditure and showed that administration of celastrol to mice housed at either room temperature or at thermoneutrality was associated with increased oxygen consumption and with elevation of brown fat gene expression programs in BAT and iWAT (Figures 5G and 5H), with no concomitant changes in food intake nor in locomotor activity (Figures S4F and S4G). The effects on oxygen consumption were recapitulated in vitro and abolished in HSF1-depleted cells, demonstrating that celastrol affects energy expenditure in a HSF1-dependent manner (Figure 5I).

Celastrol Increases Mitochondrial Function in Muscle

To determine whether the HSF1-PGC1α axis was also activated in other metabolic tissues in addition to fat, we analyzed the levels of PGC1α and its target genes in muscle, brain, kidney, and liver of celastrol-treated mice and in mice with HSF1 ablation (Figures S5A and S5B). This survey revealed that the transcriptional program downstream of PGC1α is activated also in skeletal muscle. Furthermore, we examined the expression of hypothalamic genes regulating energy balance previously reported to be altered in mice with neuronal specific knockout of PGC1α (Ma et al., 2010) and showed no changes in their expression levels in treated mice (Figure S5C).

Since it has been shown that PGC1α function in muscle contributes to energy expenditure (Handschin and Spiegelman, 2006; Lagouge et al., 2006), we further analyzed the effects of HSF1 activation on PGC1α and on its targets in mice treated with celastrol during HFD and demonstrated increased expression of mitochondrial and fiber-type genes (Figure 6A), reduced ectopic fat deposition (Figure 6B), and increased mitochondrial number in the gastrocnemius of celastrol-treated mice (Figure 6B). Given that modulation of PGC1α levels in muscle has been shown to affect exercise tolerance (Handschin et al., 2007), we tested the effects of celastrol treatment on muscle endurance. Our analysis revealed that treated mice had
increased ability to run longer, to cover a larger distance, and to generate higher average work compared to controls (Figure 6C), consistent with an overall increase in muscle endurance.

Conversely, mice with ablation of HSF1 showed reduced oxidative fibers in their gastrocnemius (Figures 6D and 6E) and impaired muscle endurance (Figure 6F). Furthermore, HSF1 gain of function in muscle was associated with increased expression of mitochondrial genes in WT but not in PGC1α KO mice, suggesting that the effects of HSF1 on muscle physiology are PGC1α dependent (Figure 6G). Overall, these data suggest a role of the HSF1-PGC1α axis also in modulating muscle physiology.

Celastrol Protects against Obesity, Insulin Resistance, and Hepatic Steatosis through HSF1 Activation

To further examine the physiological implications of the metabolic reprogramming induced by celastrol, we assessed whether HSF1 pharmacological activation would prevent obesity. As shown in Figure 7A, celastrol treatment throughout the HFD regimen protected mice from weight gain, without affecting their food intake, locomotor activity, or respiratory exchange ratio (Figure S6A). Examination of body composition by NMR spectroscopy revealed a marked and selective decrease in fat mass in celastrol-treated mice (Figures 7B and S6B). Consistent with this reduction, we observed decreased adipose depots exhibiting smaller adipocytes and reduced lipid accumulation (Figures 7C and 7D), an improvement in metabolic parameters (Figures 7E and S6B), and enhanced insulin sensitivity (Figures 7F) in the celastrol-treated group. Reductions in the liver enzyme alanine transaminase (ALT) and in the levels of the proinflammatory cytokine TNFα were also observed in the treated group (Figure S6C). Further evidence of improved metabolic profiles in celastrol-treated mice was the decrease in liver weights due to reduced hepatic steatosis and intrahepatic triglycerides accumulation (Figures 7G–7J). Interestingly, celastrol treatment of mice on regular chow diet also was associated with a reduction in adipose depots, but not with body weight changes (Figures S6D and S6E). To further ensure that the effects of celastrol on body weight were not related to compound toxicity, we monitored a number of parameters from the beginning of the treatment over a 2 week period. As shown in Figures S6F–S6J, while celastrol-treated mice showed decreased body weight and increased oxygen consumption compared to control mice, they had similar food intake and locomotor activity. Blinded analysis of H&E-stained histological tissue sections revealed no apparent pathology (Figure S6K), and serum chemistry analysis demonstrated the absence of any readily apparent drug toxicity (Table S1). As further evidence of the specificity of the compound and the genetic requirement of HSF1, celastrol treatment in HSF1 null mice had no effects on body weight, metabolic parameters, cold tolerance, or brown fat gene programs in BAT or iWAT (Figures 7J and S6L). Overall, these data demonstrate that pharmacological activation of HSF1 protects from diet-induced obesity and improves metabolic parameters in mice.

DISCUSSION

We have uncovered a critical role for HSF1 as a transcriptional regulator of brown fat, beige fat, and muscle physiology. HSF1 has been traditionally characterized as an ancient, conserved fac-

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Our data suggest that pharmacological activation of the HSF1-PGC1α axis with celastrol ameliorates metabolic dysfunction. Of note, celastrol has been known for its antioxidative and anti-inflammatory properties (Salminen et al., 2010), and reported to cross the brain-blood barrier (Kiae et al., 2005). Although our analysis did not reveal any significant changes in the levels of mitochondrial genes in the brain of treated mice, it remains to be assessed whether the beneficial effects of celastrol for the treatment of obesity are due to activation of the sympathetic nervous system in addition to its intrinsic effects on fat and muscle reported here.

Overall our study provides a proof of principle that pharmacological activation of HSF1 promotes thermogenic and mitochondrial gene programs in brown and beige cells and in skeletal muscle, with profound impact on metabolic parameters. The beneficial effects of HSF1 activation by celastrol observed in an animal model of diet-induced obesity included decreased adipose tissue expansion in response to HFD regimens and concomitant relief from the associated comorbidities, including improvement in insulin sensitivity and decreased hepatic steatosis. It is worth noting that, to date, pharmacological treatments such as those employing agonists for the β3-adrenergic receptor and for PPARγ have had little clinical success due either to their limited effects in enhancing energy expenditure (Cypess and Kahn, 2010; Cypess et al., 2012) or to dose-limiting adverse events (Boss and Farmer, 2012). In contrast, our data suggest that pharmacological activation of the HSF1-PGC1α axis may represent a possible strategy to ameliorate a wide range of pathologies associated with altered metabolic conditions.

While this paper was in revision, another report was published indicating that celastrol can act as a leptin sensitizer to counteract obesity (Liu et al., 2015). While both studies highlight the beneficial effects of celastrol in treating obesity, the two reports differ in the proposed mechanisms of celastrol’s action. Specifically, the other recent report focused on celastrol’s central effects on decreasing weight by affecting food intake, while our paper emphasizes celastrol peripheral action through activation of a transcriptional axis involving HSF1 and PGC1α. The contrasting mechanisms described in these two reports may reflect differences in the sensitivities of different tissues for different dosages of celastrol. This conclusion is bolstered by the fact that our analysis, using 3-fold fewer amounts of celastrol than recently described, did not uncover any effects on food intake. It should be noted that the two studies also differed on the models of obesity analyzed. Our studies involved normal mice fed a HFD, a condition in which celastrol was protective. In contrast, the other manuscript analyzed the effects of celastrol on obese and leptin-resistant mice. Interestingly, while the recent study primarily focused on celastrol effects on food intake, in their analysis they did observe that in pair-feeding experiments celastrol increased energy expenditure. Such results are consistent with our observations presented here. Further studies should help clarify whether central or peripheral effects of celastrol predominate at clinically relevant concentrations of the drug.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

10T1/2 cells (ATCC) and primary fat SVF cells were used in this study. A total of 90% confluent 10T1/2 cells were immediately transferred to a water bath placed inside a CO2 incubator set at 37°C or 31°C for the indicated time. For heat shock experiments, 10T1/2 cells were exposed at 42°C for 1 hr. For cooling experiments, 10T1/2 cells were transfected with siRNA, and 48 hr later medium was changed with culture medium prewarmed at 37°C or 31°C and immediately transferred to a water bath placed inside a CO2 incubator set at 37°C or 31°C for the indicated time. For the analysis of the effects of celastrol on gene expression of differentiated cells, 10T1/2 cells were treated with celastrol for 24 hr after 7 days of differentiation. The analysis of the effects of HSF1 knockdown was performed in 10T1/2 cells transfected with control or siHSF1 and differentiated for 4 days prior to treatment with 0.5 μM celastrol for 24 hr. To obtain WT, HSF1 null and PGC1α null mouse SVF, BAT, and iWAT were obtained from WT and null mice, minced, and subjected to collagenase (1 mg/ml) digestion at 37°C for 45 min in buffer containing 0.123 M NaCl, 5 mM KCl, 1.3 mM CaCl2, 5 mM glucose, 100 mM HEPES, and 4% BSA; filtered through a 100 um nylon screen; and centrifuged at 150 g for 5 min at room temperature. Cell pellets were washed twice and resuspended in DMEM medium containing 25 mM glucose, 20% FBS, 20 mM HEPES, and 1% pen/strep, and culture medium was changed daily. The differentiation process for 10T1/2 and SVF was described in Supplemental Experimental Procedures.

**Cellular Metabolic Rates**

Cellular metabolic rates were measured using a XF24 Analyzer ( Seahorse Bioscience). 10T1/2 cells were transfected with control or si-HSF1 and differentiated for 5 days and/or treated with vehicle or celastrol for 72 hr. BAT and iWAT SVF cells from WT and HSF1 null mice were differentiated for 8 days. Respiration was measured under basal conditions, following the addition of ATP synthase inhibitor oligomycin, the mitochondrial uncoupler FCCP, or the complex III inhibitor antimycin A. The calculation methods can be found in Supplemental Experimental Procedures.

**Transfections and Luciferase Assays**

10T1/2 cells were transfected with X-treme HP (Roche) or Nucleofector 96-well Transfections and Luciferase Assays. 10T1/2 cells were transfected with control or si-HSF1 and differentiated for 4 days prior to transfection with dual luciferase system (Promega). The PGC1α-luciferase reporter was purchased from Addgene. A deletion mutant of the PGC1α-luciferase reporter at the HSE putative sequence was generated with QuikChange II Site-Directed Mutagenesis Kit (Agilent), using the following primers: F, 5′-ATGGTGCTGGGT-3′, and R, 5′-CTAATTCTACTTTAAAAAAAA CTAACCCAGCCAT-3′.

**Real-Time PCR**

Total RNA was extracted from cultured cells or tissues with TRIzol (Invitrogen), and 1 μg total RNA was reverse transcribed to cDNA with First-Strand cDNA Synthesis Kit (Roche), according to the manufacturers’ instructions. Quantitative real-time PCR was performed with the ABI PRISM 7900HT sequence.
detection system (ABI) using SYBR green (Roche). The details can be found in Supplemental Experimental Procedures.

**Coimmunoprecipitation Assay and Protein Analysis**

Coimmunoprecipitation was performed with the Catch and Release v2.0 Reversible Immunoprecipitation System (Millipore). Proteins were separated by 10% Bis-Tris Gel (Invitrogen), transferred to PVDF membranes (Pierce), and incubated with anti-PGC1α (Santa Cruz, sc-13067) or anti-HSF1 (Abgent, AJ13744a) antibodies overnight at 4°C and at room temperature for 1 hr with secondary antibodies. Immune complexes were visualized by using SuperSignal West Dura Extended Duration Substrate (Pierce), following the manufacturer’s instructions. For measuring UCP1 protein levels in tissues with western blot analysis, proteins were extracted with RIPA buffer consisting of 20 mM Tris, 150 mM NaCl, 1% Triton X-100, and protease inhibitors (Roche), and blot analysis, proteins were extracted with RIPA buffer consisting of 20 mM Tris, 150 mM NaCl, 1% Triton X-100, and protease inhibitors (Roche), and 15 μg proteins were loaded on a 10% Bis-Tris Gel and transferred to PVDF membranes (Pierce) and incubated with anti-UCP1 (Abcam, ab10983) and anti-β-actin (Sigma, A5316) antibodies.

**Chromatin Immunoprecipitation Assays**

ChIP assays were performed using a ChIP assay kit (Millipore), according to manufacturer’s instructions and as previous reported (Ma et al., 2014); details can be found in Supplemental Experimental Procedures.

**Animal Studies**

Mouse studies were performed according to guidelines of the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee. C57BL/6J, HSF1 heterozygous mice (Balb/C) were purchased from The Jackson Laboratory. PGC1α heterozygous mice were a kind gift of Bruce M. Spiegelman (Harvard Medical School, Boston, MA, USA). Heterozygous mice were crossed to obtain WT and knockout genotypes. Male mice were used for all the experiments described in this study. For treatments, celastrol was mixed with powdered chow (NIH 07) or with HFD chow (60%, ResearchDiet, D12492). For assessing metabolic parameters, 2-month-old mice were fed either chow diet or HFD with or without celastrol for 3 weeks and 8 weeks. Body composition was measured using NMR (Echo Medical Systems). To monitor the effects of celastrol treatment, 2-month-old mice were fed HFD with or without celastrol for 2 weeks, and body weights, oxygen consumption, food intake and locomotor activity were measured every 2 days throughout the experiments with CLAMS (CLAMS Instruments, Columbus, OH, USA). To measure the metabolic effects of celastrol on WT and HSF1 KO mice, 2-month-old mice were fed a HFD, with or without celastrol, for 4 weeks, and parameters such as body weight, insulin sensitivity, liver weights, and cold tolerance were measured at the end of the treatment. The details for cold exposure, treadmill exercise, energy expenditure, and GTT and ITT analysis can be found in Supplemental Experimental Procedures.

**Adenoviral Delivery in Inguinal Fat and Muscle**

Adenoviruses expressing control (CMV-GFP), mouse HSF1 (HSF1-CMV-GFP), control shRNA (U6-shRNA-CMV-GFP), shHSF1 (U6-shHSF1-CMV-GFP), and shPGC1α (U6-shPGC1α-CMV-GFP) were constructed, amplified, and purified by Vector Biolabs. A total of 50 μl of 5 x 10^6 Pu of each adenovirus diluted in saline was injected bilaterally (s.c.) into the inguinal fat pads of mice (Ma et al., 2014). For intramuscular delivery of adenovirus, 10 μl of 2 x 10^10 Pu/μl control or mouse HSF1 adenovirus diluted in saline was injected unilaterally into the contralateral lower limbs of mice (Rias et al., 2012). Mice were either sacrificed at the fourth day after viral delivery or were celastrol treated as noted, and tissues were dissected for further analysis or monitored for changes in body temperature under cold environment for 3 hr.

**Serum Parameters and Liver Triglyceride Level Determination**

Serum leptin and insulin levels were measured with RIA kits (Millipore); blood glucose levels were measured with an automated reader (Bayer); triglyceride (Thermo), cholesterol (Sigma), HDL (Sigma), LDL/VLDL (Sigma), and ALT (Sigma) levels were assayed by colorimetric tests; and serum TNFα (R&D) and IFN-γ (Phoenix Pharmaceuticals) were determined by ELISA. Analysis of serum chemistry including levels of serum minerals, lipids, and renal and liver enzymes was performed by the Department of Laboratory Medicine at the NIH Clinical Center. Lipids were extracted from liver samples, and triglyceride levels were determined by triglyceride reagent (Sigma). The details can be found in Supplemental Experimental Procedures.

**Histological Analysis**

Dissected tissues were fixed in 10% neutral buffered formalin and embedded in paraffin according to standard procedures. Tissue sections of 5 μm thickness were stained with H&E (Histoserv) or with UCP1 antibody (Abcam, ab10983), following the manufacturer’s instructions (Vector labs). Frozen sections of gastrocnemius muscle from WT and HSF1 KO mice were prepared and stained with succinic dehydrogenase (SDH). Transmission electron microscopy (TEM) analysis was performed by EM Facility, National Cancer Institute, at Frederick, MD, USA. The details can be found in Supplemental Experimental Procedures.

**Human Adipose Tissue**

Fat biopsies of subcutaneous abdominal region were obtained from three obese women (BMI >40) recruited at the NIH. The study was approved by the NIDDK Institutional Review Board (clinicaltrials.gov identifier NCT00428987). All subjects gave written informed consent. SVF cells from human subcutaneous fat tissue were obtained, differentiated, and treated with DMSO or celastrol at the concentrations indicated for 24 hr. The details can be found in Supplemental Experimental Procedures.

**Statistical Analysis**

Student’s t test was used for comparison between two groups. One-way ANOVA followed by the Dunnett post hoc test was used for multiple comparisons versus the control group (GraphPad Software). Two-way ANOVA was used to examine interactions between variables and ANCOVA was used to analyze oxygen consumption data of WT and HSF1 null mice by SPSS software (Tischöp et al., 2011), p < 0.05 was considered as statistically significant. Results are shown as mean ± SEM.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2015.08.005.

**AUTHOR CONTRIBUTIONS**

X.M., L.X., and E.M. conceived the project. X.M. performed biochemical and cellular experiments, and L.X. performed animal experiments. A.T.A. performed the human adipocyte experiments. O.G. analyzed energy expenditure. A.B. performed preliminary experiments. M.S. recruited obese participants. J.L. assisted with measurement of cellular metabolic rates. T.F. participated in the experimental design and the interpretation of results. X.M., L.X., and E.M. wrote the manuscript. O.G. and T.F. edited the manuscript. E.M. devised the project, supervised, and coordinated the execution of the experimental plan.

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