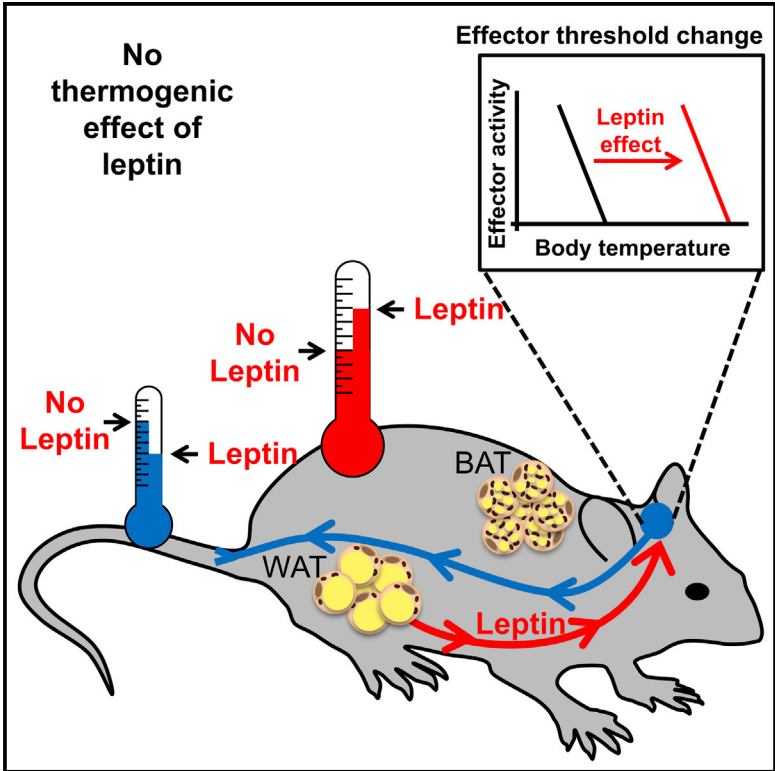# Cell Reports

Leptin Raises Defended Body Temperature without Activating Thermogenesis

Graphical Abstract

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In Brief

Leptin is thought to reduce body weight by both reducing food intake and increasing brown adipose tissue (BAT) thermogenesis. In the present study, Fischer et al. show that leptin does not affect BAT thermogenesis; instead, it leads to a pyrexic increase in body temperature by reducing heat loss.

Highlights

- ob/ob mice are not hypometabolic and do not have reduced BAT thermogenic capacity

- ob/ob mice are not hypothermic but display downward shift in thermoregulatory thresholds

- Leptin is not thermogenic and does not increase BAT recruitment

- Leptin is pyrexic and increases body temperature by reducing heat loss

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Leptin Raises Defended Body Temperature without Activating Thermogenesis

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SUMMARY

Leptin has been believed to exert its weight-reducing action not only by inducing hypophagia but also by increasing energy expenditure/thermogenesis. Leptin-deficient ob/ob mice have correspondingly been thought to be thermogenically limited and to show hypothermia, mainly due to atrophied brown adipose tissue (BAT). In contrast to these established views, we found that BAT is fully functional and that leptin treatment did not increase thermogenesis in wild-type or in ob/ob mice. Rather, ob/ob mice showed a decreased but defended body temperature (i.e., were anapyrexic, not hypothermic) that was normalized to wild-type levels after leptin treatment. This was not accompanied by increased energy expenditure or BAT recruitment but, instead, was mediated by decreased tail heat loss. The weight-reducing hypophagic effects of leptin are, therefore, not augmented through a thermogenic effect of leptin; leptin is, however, pyrexic, i.e., it alters centrally regulated thresholds of thermoregulatory mechanisms, in parallel to effects of other cytokines.

INTRODUCTION

A large number of physiological effects are attributed to leptin (Friedman, 2014). However, it is in its role as regulator of body weight that leptin has accrued the most interest. Particularly, there is a general notion that leptin reduces body weight through both a reduction of food intake and an increase in thermogenesis/energy expenditure (EE) (Cannon and Nedergaard, 2004; Haynes, 2000; Himms-Hagen, 1999; Kalil and Haynes, 2012; Mantzoros, 1999; Mark, 2013; Park and Ahima, 2015; Rahmouni, 2010; Rezai-Zadeh and Münzberg, 2013; Simonds et al., 2012). Whereas a multitude of investigations have, over the years, detailed the pathways involved in the hypophagic effect of leptin, only a few studies have addressed the suggested thermogenic pathway (Breslow et al., 1999; Commins et al., 1999; Hwa et al., 1997; Morrison, 2004; Scarpace et al., 1997).

That leptin should be thermogenic is based, e.g., on observations that the absence of leptin (as in the ob/ob mice) has been associated with hypometabolism and hypothermia (Bray and York, 1979; Kaiyala et al., 2015; Pelleymounter et al., 1995) and with brown adipose tissue (BAT) inactivation (Ashwell et al., 1985; Bas et al., 1983; Himms-Hagen and Desautels, 1978). Accordingly, one effect of leptin would be to induce thermogenesis by the activation of BAT and, through this, to counteract hypothermia and promote body weight loss. However, we conclude here that the protective effects of leptin in body weight regulation do not involve an increase in EE.

RESULTS

No Acute Thermogenic Response to Leptin

To investigate whether leptin—by, e.g., activation of the sympathetic nerves (Kalil and Haynes, 2012) innervating BAT—has a direct, acute thermogenic effect, we examined the thermogenic response to acute leptin treatment.

First, we verified the ability of BAT to respond to a thermogenic stimulus by performing indirect calorimetry on wild-type (WT) C57BL/6 mice injected with the β₃-agonist CL316,243. As seen (Figure 1A), treatment with CL316,243 caused a marked increase in oxygen consumption. However, different doses of leptin (as used in the literature) did not cause any increase in oxygen consumption (Figure 1B). Thus, at least under these standard conditions, leptin was not an acute thermogenic agent.

The aforementioned experiments were performed in pentobarbital-anesthetized mice. Although pentobarbital—in contrast to, e.g., isoflurane—does not inhibit BAT thermogenesis (Ohlson et al., 2003, 1994; Virtue and Vidal-Puig, 2013), we further analyzed the effects of acute leptin injections on EE in conscious, ambulatory WT mice. Also, under these conditions, leptin injections did not increase EE (Figure 1C).

To exclude the possibility that the absence of a thermogenic effect of leptin may be due to an already saturated leptin system in WT mice (Leibel, 2002), we injected the leptin-deficient ob/ob mice with leptin. Leptin treatment of ob/ob mice did not alter EE
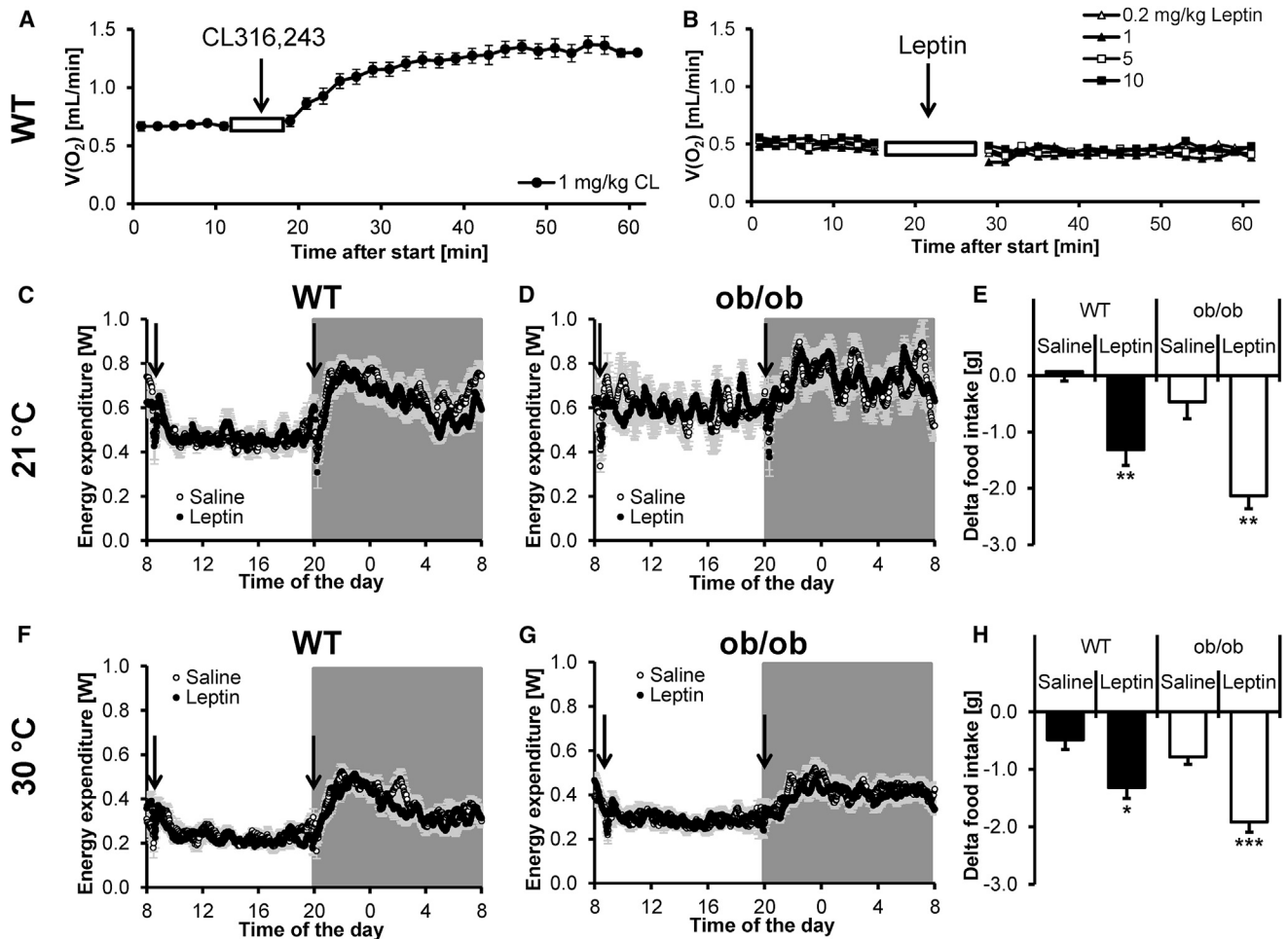
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Figure 1. No Acute Thermogenic Response to Leptin
Male WT mice were injected with the β₃-agonist CL316,243 or saline or different doses of leptin to measure acute thermogenic effects of leptin.
(A) Acute effect of 1 mg/kg CL316,243 (CL) on oxygen consumption (V(O₂)) in anesthetized male WT mice. n = 6.
(B) Acute effect of different doses of leptin on oxygen consumption in anesthetized male WT mice; n = 2 for each dose; SEM not shown.
(C and D) Effects of 5 mg/kg leptin on EE in conscious WT mice (C) and ob/ob mice (D) housed and measured at 21°C. Arrows indicate injections.
(E) Effects of leptin treatment on food intake at 21°C. Food intake was measured during 24 hr before treatment and 24 hr after injections. The difference in 24-hr food intake between pre-treatment and post-treatment levels is shown. Pre-treatment levels (g/24 hr) were: WT saline, 4.5 ± 0.2; WT leptin, 4.3 ± 0.2; ob/ob saline, 4.7 ± 0.3; ob/ob leptin, 4.2 ± 0.2.
(F and G) Effects of 5 mg/kg leptin on EE in WT mice (F) and ob/ob mice (G) housed at 21°C and measured at 30°C.
(H) Effects of leptin treatment on food intake at 30°C. Pre-treatment levels were: WT saline, 3.2 ± 0.2; WT leptin, 3.1 ± 0.1; ob/ob saline, 3.3 ± 0.2; ob/ob leptin, 3.2 ± 0.1.
In (C)–(H), n = 5–6. Data indicate means ± SEM. *p ⩽ 0.05; **p ⩽ 0.01; ***p ⩽ 0.001, significant differences between treatments calculated using Student's t test. See also Figure S1.

(Figure 1D). The lack of effect was not age dependent, as we also failed to detect thermogenic effects of leptin in 7- to 8-week-old ob/ob mice (Figure S1B). To validate the efficacy of the leptin injections, we measured its effect on food intake in both WT and ob/ob mice. As expected, leptin treatment resulted in a significant reduction of food intake, as compared to saline-treated controls (Figure 1E).

At environmental temperatures below thermoneutrality, possible effects of thermogenic agents can be masked by a compensatory decrease in thermoregulatory thermogenesis, i.e., the heat production occurring in order to defend the body

temperature. This makes the agent-induced thermogenic effect invisible (Goldgof et al., 2014; Nedergaard and Cannon, 2014; Shemano and Nickerson, 1963). Therefore, the experiments were repeated at thermoneutrality, i.e., within the environmental temperature zone where no heat-production is needed to compensate for heat loss to maintain stable body temperature. However, leptin treatment of WT or ob/ob mice at 30°C again did not lead to any significant increase in EE (Figures 1F, 1G, and S1C), while the effects on food intake were maintained (Figure 1H). Thus, our results demonstrate the absence of an acute thermogenic effect of leptin.
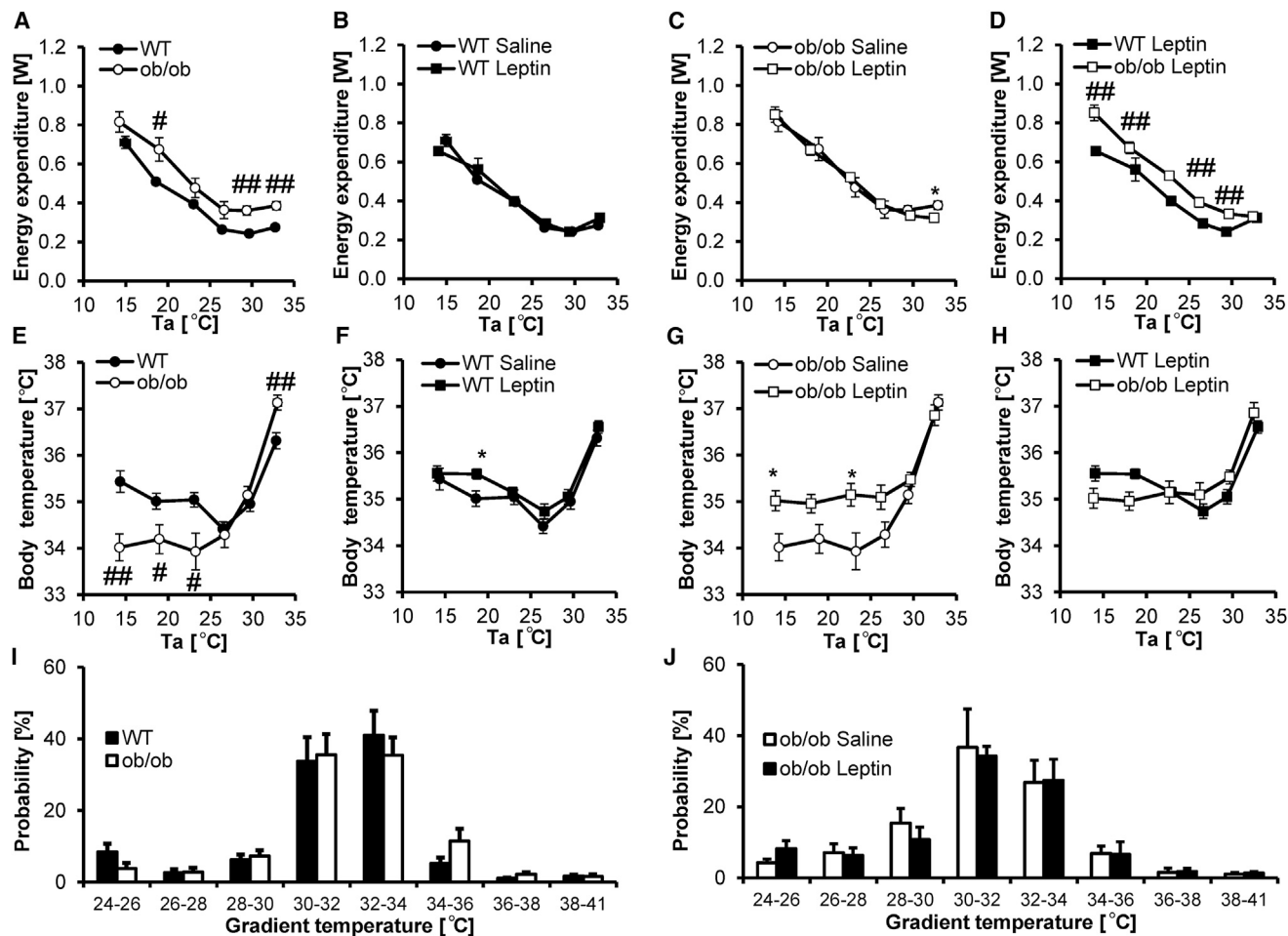
Figure 2. Leptin Affects the Defended Tb at Subthermoneutral Temperatures

(A–H) Male WT (body weight = 27.4 ± 0.5 g) and ob/ob (body weight = 48.1 ± 1.0 g) mice were exposed to different environmental temperatures from 33°C to 16°C. In (A)–(D), Scholander plots of WT mice treated with saline (●) or leptin (■) and ob/ob mice treated with saline (○) or leptin (□) are shown. The values are average EE for the last hour at each temperature. (E–H) Tb within the same time frame. Note that the data presented in (A) and (E) and (D) and (H) are a reorganization of the data presented in (B) and (F) and (C) and (G). Ta, ambient temperature.

(I and J) For thermal preference tests, male WT and ob/ob mice, injected with saline or leptin, were allowed to move freely in a temperature gradient tunnel during the day, and the position of the mouse was recorded. (I) Probability distribution of WT (black) and ob/ob (white) mice for staying within pre-defined 2°C regions of the gradient. (J) Probability distribution of ob/ob mice injected with saline (white) or 5 mg/kg leptin (black) 24 hr and 2 hr before start of the experiment.

n = 5–7. Data indicate means ± SEM. *p ≤ 0.05; significant differences between treatments calculated using Student's t test. #p ≤ 0.05; ##p ≤ 0.01; significant differences between genotypes calculated using Student's t test.

See also Figure S2 and Movie S1.

ob/ob Mice Are Not Hypometabolic

As a corollary to the tenet that leptin is thermogenic, ob/ob mice are generally described as being hypometabolic (see Introduction). We performed modified Scholander experiments (Scholander et al., 1950) to measure the temperature dependence of the effects of leptin deficiency and leptin treatment on EE, body temperature (Tb), and locomotor activity. No effects of genotype or treatment on locomotor activity were observed (Figures S2A–S2D).

WT mice (Figure 2A, filled symbols) displayed stable EE levels at environmental temperatures between 33°C and 27°C (Figure 2A). This temperature range, thus, represents the thermoneutral zone. At environmental temperatures below 27°C, the

EE of the WT mice increased linearly with decreasing environmental temperature, representing the need to perform thermogenesis to compensate for the increasing heat loss (Figure 2A). The slope of the increase in EE is a function of the insulation of the animal, since better insulation reduces the need for heat production (Scholander et al., 1950).

In ob/ob mice (open symbols), the thermoneutral zone was also 27°C–33°C (Figure 2A). Remarkably, ob/ob mice showed significantly higher EE than WT mice. This finding was surprising, as others have reported the metabolic rates of ob/ob mice to be significantly lower than those of WT mice (Pelleymounter et al., 1995). Indeed, this has been suggested to contribute to their obese phenotype. However, the Pelleymounter data were

normalized per kilogram of body weight rather than per mouse (see Discussion). In Figures S2E and S2F, we have plotted the individual EE data versus body weight and, as recommended by Tschöp et al. (2012), versus lean mass. As seen, the ob/ob mice are clearly hypermetabolic when analyzed in this way, demonstrating higher EE despite lower lean mass (Figure S2I).

As the slope of the increase in EE in the Scholander experiments was not different between WT and ob/ob mice, a defect in insulation was not driving increases in EE. The mice did not show significant differences in food intake, as compared to WT controls (Figure S2J), which is most likely a reflection of their age; ob/ob mice show only a transient hyperphagic phase (Figure S1A) (Hanson and Morton, 1983). Thus, the explanation for the higher EE of the ob/ob mice cannot be a higher thermic effect of food as a result of hyperphagia.

ob/ob Mice Are Not Hypothermic

It is generally assumed that leptin-deficient ob/ob mice display hypothermia, i.e., they are unable to fully defend Tb due to the (unsustainable) tenet that they are hypometabolic. Therefore, we followed Tb during the Scholander experiments. At subthermoneutral temperatures, the Tb of the WT mice (Figure 2E, filled symbols) remained, as expected, principally stable at ∼35°C (Figure 2E).

At thermoneutrality (30°C), the Tb of ob/ob mice was indistinguishable from that of WT mice (Figure 2E). At subthermoneutral temperatures (below 27°C) (Figure 2E), the ob/ob mice displayed Tbs that were lower than those of WT mice (despite having higher EE) (Figure 2A). Thus, they appear hypothermic, i.e., they appear to be in a state where they can no longer produce sufficient heat to compensate for heat loss. However, this was clearly not the case. First, the Tb was stable, even as the cold stress was further increased (Figure 2E); second, the mice were able to increase their metabolic rates further with the decreasing environmental temperature (Figure 2A). Since Tb remained stable at subthermoneutral temperatures, and the mice were fully able to defend this lower Tb by increased metabolism, the ob/ob mice at subthermoneutral temperatures are not hypothermic but rather anapyrexic (i.e., they defend a lower Tb). Thus, leptin would appear to have an effect on the central control of Tb.

Leptin Normalizes the Defended Tb Setting

Since ob/ob mice displayed a lower Tb than WT mice at subthermoneutral temperatures, the effects of leptin treatment on EE and Tb were analyzed in these mice. Leptin treatment of WT mice did not lead to significant changes in EE (Figure 2B) but to a slight increase in Tb at subthermoneutral temperatures (Figure 2F). Correspondingly, during a 24-hr recording, leptin-treated WT mice displayed a slight increase in Tb at 21°C during the light phase (Figure S1D), not paralleled by an increased EE (Figure 1C), while at 30°C, no Tb effect was observed in WT mice during acute recording (Figure 2F) and 24-hr recording (Figure S1G).

Leptin treatment of ob/ob mice did not increase EE at any temperature (Figure 2C). Despite not increasing EE, leptin treatment of ob/ob mice led to a robust elevation of Tb at all subthermoneutral temperatures (Figure 2G). Indeed, leptin treatment of ob/ob mice led to Tbs statistically indistinguishable from those of WT

mice (Figure 2H) but did not eliminate the difference in EE between the WT and ob/ob mice (Figure 2D). Correspondingly, during a 24-hr recording, the leptin-treated ob/ob mice at 21°C displayed an increased Tb both during the light phase and during most of the dark phase (Figure S1E), again, not paralleled by an increased EE (Figure 1D). At 30°C, no effect of leptin treatment on Tb was seen during the 24-hr recording (Figure S1H).

Leptin Counteracts the Development of Torpor

Due to the relationship between leptin and food intake, we examined whether the absence of food during the experiments would affect EE. Even in fasted mice, there was no significant effect of leptin on EE, neither at 30°C nor at 21°C, and neither in wild-type nor in ob/ob mice (Figures S2G and S2H). The tendency to an increase in EE in ob/ob mice at 21°C probably merely represents a counteraction of fasting-induced torpor (discussed in the following text).

In an extension of this, we pair-fed ob/ob mice to ob/ob mice that received leptin injections and thus reduced their food intake. As expected, during the light phase, leptin treatment prevented the pair-feeding-induced drop in Tb (Figures S1E, S1F, S1H, and S1I). The effect of leptin during the dark phase was much more pronounced. In agreement with earlier data (Himms-Hagen, 1985; Webb et al., 1982), the pair-fed mice, in the relative cold of 21°C and in the absence of sufficient food, went into torpor at the end of the night: the Tb dropped conspicuously (Figure S1E). The much-lowered Tb resulted necessarily in a diminished metabolic rate (Figure S1F). However, through its pyrexic effect, leptin prevented the torpor; the mice defended their Tb; and there was, accordingly, no decrease in metabolic rate. Mice at thermoneutrality could, for obvious reasons, not display full torpor, but the metabolic pattern was similar (Figures S1H and S1I). Thus, leptin acted by maintaining the Tb at a euthermic level counteracting torpor induction (Bolze et al., 2015; Döring et al., 1998; Gavrilova et al., 1999; Stehling et al., 1996).

Thermal Preference Behavior Is Not Affected by Leptin

The stable but reduced Tb in ob/ob mice at subthermoneutral temperatures and the immediate correction of this phenotype after leptin treatment could be explained by a shift in Tb set-point. In that case, the ob/ob mice should also defend the lower Tb at higher environmental temperatures. This does not seem to be the case (Figure 2E). However, the concept of a set-point is controversial. There may not be a central set point but, rather, an array of effector mechanisms with different thresholds (McAllen et al., 2010; Romanovsky, 2004), which results in the apparent existence of a common set point. Therefore, we examined whether other thermoregulatory effectors were also affected by leptin. For this, we used a thermal preference test. In this test, the mice, during daytime, are allowed to move freely in a channel with a defined temperature gradient to choose their preferred temperature; thus, they utilize behavioral rather than metabolic thermoregulation (Movie S1).

The experiments revealed no measurable differences between WT and ob/ob mice (Figure 2I), indicating no inherent difference in the preferred temperature. Similarly, leptin replacement in ob/ob mice did not alter the thermal preference behavior (Figure 2J). This lack of difference in preferred temperature between

WT and ob/ob mice is parallel to findings in the Scholander tests, showing the same range of the thermoneutral zone in both genotypes.

ob/ob Mice Do Not Have Reduced BAT Thermogenic Capacity

Based on the tenet that ob/ob mice are hypometabolic and hypothermic (a tenet that, according to the studies discussed earlier, is not sustainable), a failure in the thermogenic processes in these mice has been suggested (see Introduction). As all adaptive, facultative, non-shivering thermogenesis is located to BAT (Golozoubova et al., 2001), any failure in the capacity for non-shivering thermogenesis must be located in BAT. Analysis of interscapular BAT (IBAT) revealed a significantly higher tissue weight in ob/ob mice (Figure 3A), but with a lower protein density (Figure 3B), resulting in the total protein content of the tissue being the same in WT and ob/ob mice (Figure 3C). Thus, the increase in tissue weight reflects a massive accumulation of lipids in ob/ob BAT but does not demonstrate tissue atrophy.

To analyze the ability of BAT to perform non-shivering thermogenesis, uncoupling protein 1 (UCP1) levels in WT and ob/ob mice were measured. This revealed essentially equal amounts of UCP1 per milligram of protein in the two genotypes (Figures 3D and 3F). Since the IBAT protein content and the amount of UCP1 per milligram of protein were similar, the total UCP1 levels in the total IBAT depot were the same in WT and ob/ob mice (Figure 3G) (despite lower UCP1 content per milligram of tissue; Figure 3E). This parameter, i.e., the total amount of UCP1 in the tissue, is generally accepted to be the most relevant biochemical parameter for non-shivering thermogenic capacity of BAT (Cannon and Nedergaard, 2004; Virtue and Vidal-Puig, 2013). Thus, the absence of leptin does not decrease non-shivering thermogenic capacity.

We also directly assessed the non-shivering thermogenic capacity of BAT in ob/ob versus WT mice, using the sympathetic neurotransmitter norepinephrine. These experiments revealed a similar response to norepinephrine in WT and ob/ob mice (Figure 3H), which is, thus, in accordance with the similar levels of total UCP1 protein (Figure 3G).

Therefore, the only difference between BAT in WT and ob/ob mice is the large lipid accumulation in the ob/ob mice. Lipid deposition is usually a reflection of inactive BAT, but the lack of BAT atrophy observed in the ob/ob mice (i.e., the absence of decreased UCP1 levels) does not support an inactive state. Rather, it would seem that the total lipid content of the ob/ob mice is so high that lipid is forced into all tissues—and the rate of combustion in BAT is inadequate to counteract this.

Leptin Treatment Reduces Tail Heat Loss in ob/ob Mice

Since Tb in ob/ob mice after leptin treatment at subthermoneutral temperatures was increased without EE being increased, we speculated that a decreased heat loss could generate the increase in Tb. The tail is a major thermoregulatory organ in the mouse, being very important for heat dissipation (Gordon, 1990; Warner et al., 2013). Thus, changes in tail surface temperature, indicating vasoconstriction or vasodilation, can be used as a readout for changes in heat loss. Therefore, we used infrared thermography to measure the tail temperature.

Leptin treatment of WT mice did not significantly influence tail temperature. However, in ob/ob mice, leptin treatment resulted in a significant decrease in tail temperature (Figures 3I and 3K). This effect was evident also when tail temperature was normalized to Tb (Figure 3L). Thus, an increased tail vasoconstriction and, thus, a decrease in heat loss contributed to or fully explained the increase in Tb in ob/ob mice following leptin injections at subthermoneutral environmental temperatures. Thus, a robust increase in Tb occurred in the absence of any increased EE.

Prolonged Leptin Treatment Is Markedly Pyrexic

In the aforementioned studies, where acute effects of leptin were followed, increased Tb was observed in ob/ob mice, but not in WT mice, following leptin injections at subthermoneutral temperatures. An increased Tb results in a larger difference between the environmental temperature and the Tb, thus principally increasing the magnitude of the cold stress. To elucidate whether this increase in cold stress would eventually lead to a higher degree of BAT recruitment, we treated ob/ob mice twice daily with leptin for 5 days. The experiments were carried out both at 21°C, where we expected to see effects on Tb, and at 30°C, where no effect was expected. As no effects of leptin on Tb were observable in WT mice, such mice were not examined here.

At 21°C, the saline-treated ob/ob mice showed large circadian fluctuations of Tb (Figure 4A). Leptin treatment significantly increased Tb within the first light phase of the treatment (Figure 2; Figures 4A and 4B). During the whole treatment period, Tb was increased in the leptin-treated group, and the daily amplitude was reduced (Figures 4A–4C). With time, both light-phase and dark-phase Tb in the leptin-treated mice approached a plateau-like state, with light-phase Tb being ~2.5°C higher and dark-phase Tb being ~1.5°C higher than in the saline controls. Despite this robust increase in Tb, leptin treatment again did not lead to significant changes in EE (Figures 4D and 4E).

At 30°C, leptin treatment did not cause any acute changes in Tb of ob/ob mice (Figure 2; Figures 4F–4H). However, after some days, Tb was consistently about 0.5°C higher than in those mice treated with leptin at 21°C. Leptin treatment acutely (but transiently) reduced EE (Figures 4I and 4J) and thermal conductance (Figures S4A and S4D). The decrease was not due to a decreased thermic effect of food as a result of reduced food intake, since food intake remained low throughout the entire treatment period (Figure S3E).

During the chronic leptin treatment, we observed the expected changes in body weight, body composition, food intake, and locomotor activity (Figure S3).

Prolonged Leptin Treatment Does Not Recruit BAT in ob/ob Mice

The prolonged leptin treatment of ob/ob mice resulted in a decrease in IBAT tissue weight (Figure 5A). At 21°C, leptin treatment of ob/ob mice resulted in a massive decrease in lipid droplet size and number in IBAT, as assessed by histological and protein analysis (Figures 5B and S4G). At 30°C, there were minor, and less homogeneous, effects of leptin treatment on BAT morphology. The total protein content of the IBAT was reduced by the prolonged leptin treatment at both environmental temperatures (Figure 5C).
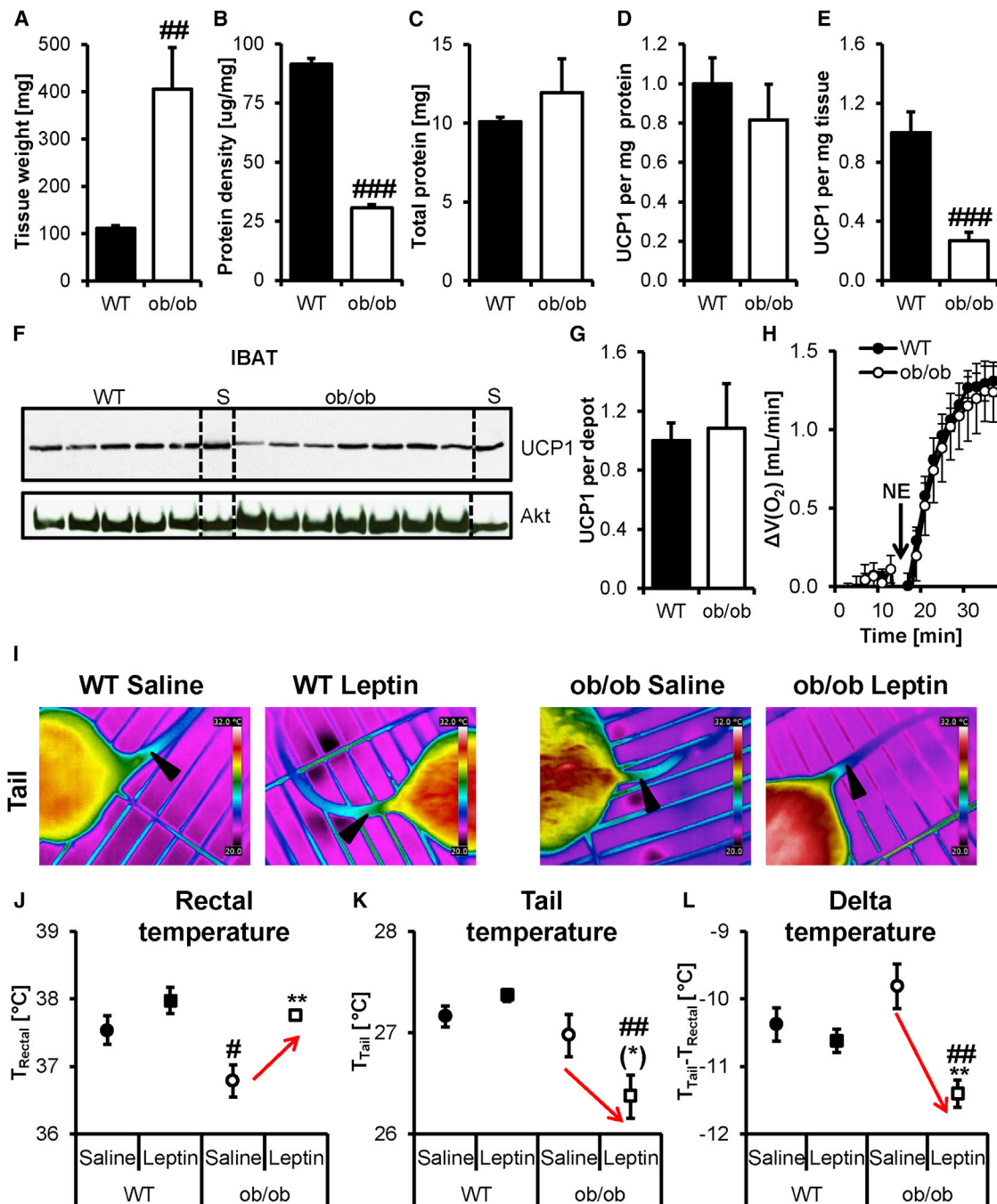
Figure 3. ob/ob Mice Do Not Display Reduced BAT Thermogenic Capacity, and Leptin Treatment Reduces Tail Heat Loss in ob/ob Mice

(A–H) WT mice (black) and ob/ob mice (white) housed at 21°C were analyzed. (A) Tissue weight of IBAT. (B) IBAT protein density. (C) IBAT total protein content, calculated as (A) × (B). (D) Quantification of UCP1 levels shown in (F); WT was set to 1. (E) UCP1 per milligram of tissue calculated from (B) and (E); WT was set to 1. (F) UCP1 western blot analysis of 20 μg IBAT protein lysate samples. A pooled IBAT lysate was used routinely as standard (S) on all blots. Total protein kinase B (Akt) was used as a loading control. (G) Total UCP1 content per IBAT depot, calculated as (C) × (E); WT was set to 1. (H) Functional analysis of BAT thermogenic capacity using the norepinephrine (NE)-injection test in animals anesthetized with pentobarbital. Increase over baseline after norepinephrine injection is shown in milliliters per minute. Baseline levels were 0.68 ± 0.02 for WT and 0.76 ± 0.08 for ob/ob.

(I–L) Infrared thermography analysis. (I) Tail temperatures measured 0.5 cm from the tail base of WT and ob/ob mice treated with saline or leptin. Arrows indicate the region of interest. (J) Rectal temperatures of WT and ob/ob mice. (K) Compilation of the results of experiments as those shown in (I). (L) The differences between tail temperatures and rectal temperatures. In (J)–(L), red arrows indicate a significant leptin effect.

For (A)–(G): n = 5–7; for (H), n = 4–5; for (I)–(L), n = 5–7. Data indicate means $\pm$ SEM. (*)$p < 0.1$ **$p \leqslant 0.01$, significant differences between treatments calculated using Student's t test. #$p \leqslant 0.05$; ##$p \leqslant 0.01$; ###$p \leqslant 0.001$, significant differences between genotypes calculated using Student's t test.
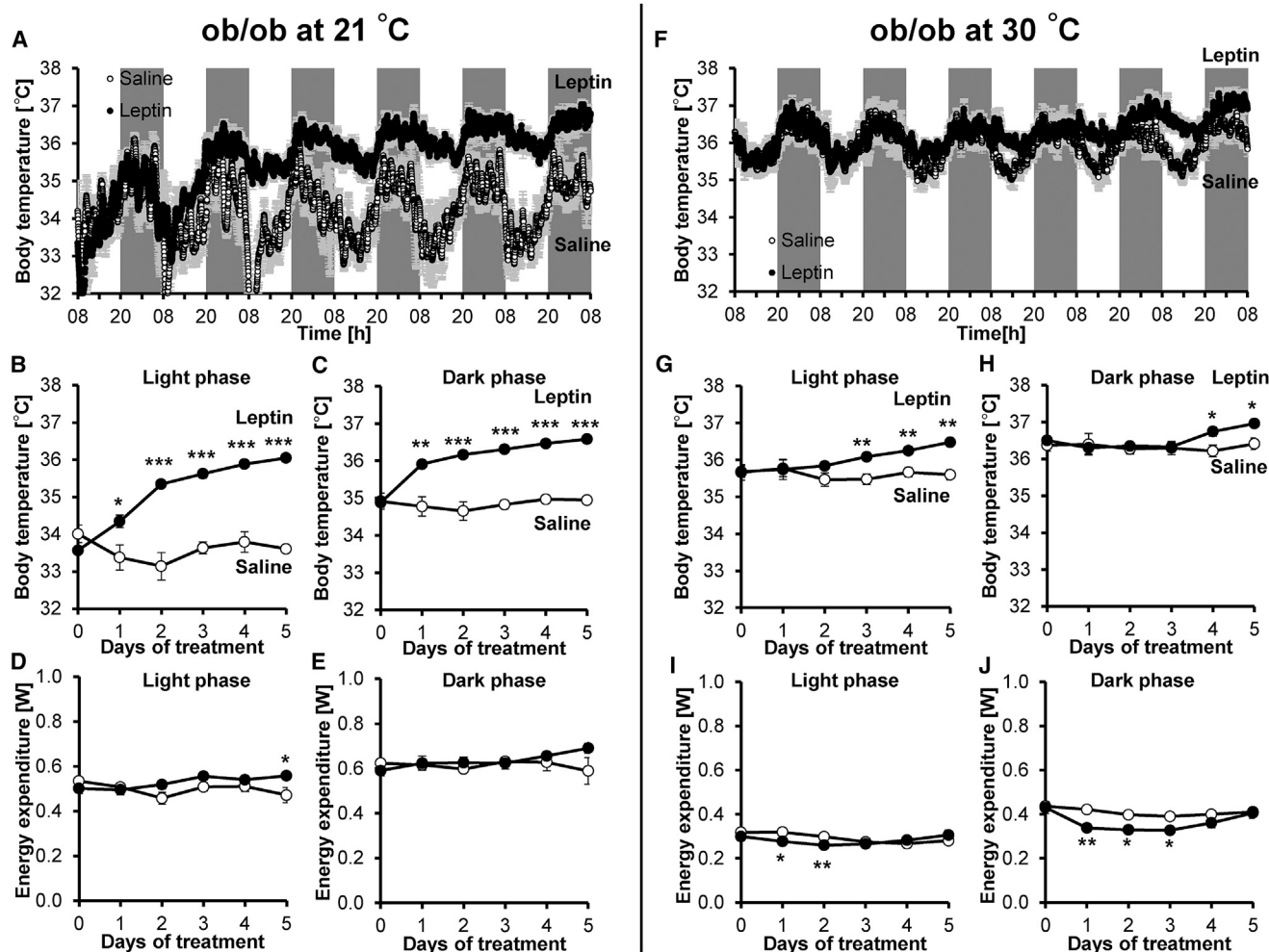
Figure 4. Prolonged Leptin Treatment Is Markedly Pyrexic

Male ob/ob mice were exposed to 21°C or 30°C in the indirect calorimetry system and treated twice daily with saline or 2.5 mg/kg leptin for 5 days, starting after 24 hr of acclimation to the calorimetry chambers. Tbs (A–C, F–H) and EE (D, E, I, and J) were followed. Data indicate means ± SEM; n = 5. *p ⩽ 0.05; **p ⩽ 0.01; ***p ⩽ 0.001, significant differences between treatments calculated using Student's t test.

See also Figures S3 and S4.

The prolonged leptin treatment at 21°C significantly induced UCP1 levels per milligram of protein (Figures 5D and 5F). However, since the total protein content of the IBAT was significantly reduced (Figure 5C), there was no significant difference between saline- and leptin-treated animals in total UCP1 per IBAT depot (Figure 5G). Also at 30°C, UCP1 levels remained unchanged (Figures 5E and 5G).

Thus, the biochemical data imply an absence of BAT recruitment due to prolonged leptin treatment. To investigate whether this was reflected physiologically, we performed norepinephrine-injection experiments in the chronically leptin-treated mice. Leptin treatment did not influence the magnitude of the norepinephrine-induced oxygen consumption, neither at 21°C nor at 30°C (Figures 5H and 5I). These results are, thus, in agreement with the observed absence of any increase in total BAT UCP1 content (Figure 5G).

DISCUSSION

In the present study, we found that leptin-deficient ob/ob mice are neither hypometabolic nor hypothermic. Analysis of BAT recruitment and thermogenic capacity revealed no defect in ob/ob mice. Leptin administration to WT and ob/ob mice did not lead to a thermogenic response; leptin treatment of ob/ob mice resulted in a defended increase in Tb that was probably mediated by reduced heat loss through tail vasoconstriction. Thus, leptin is not thermogenic but is pyrexic.

Leptin-Deficient Mice Are Not Hypo- but Rather Hypermetabolic

That ob/ob mice should be hypometabolic, and that this phenomenon—in addition to their hyperphagia—causes their obese phenotype, has mainly been based on the report that ob/ob mice display lower EE when this is normalized by body weight and that
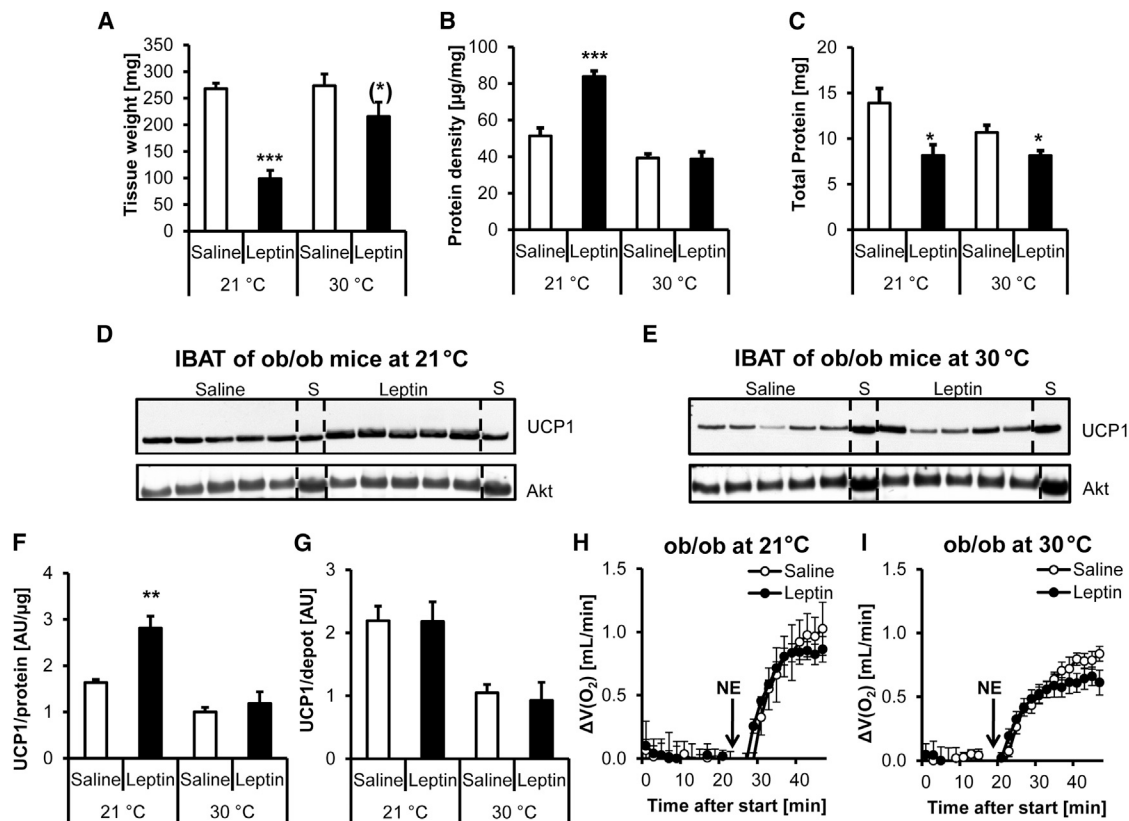
Figure 5. Prolonged Leptin Treatment Does Not Recruit BAT in ob/ob Mice

(A–I) BAT thermogenic capacity of the mice examined in Figure 4 was analyzed as described in Figure 3. UCP1 content (A–G) as well as norepinephrine (NE) response (H and I) were measured. (H and I) Baseline at 21°C: saline, 0.74 ± 0.09; leptin, 0.56 ± 0.03. Baseline at 30°C: saline, 0.52 ± 0.02; leptin, 0.56 ± 0.1. n = 4–5. Data indicate means ± SEM. (*) p < 0.1 *p ⩽ 0.05; **p ⩽ 0.01; ***p ⩽ 0.001, significant differences between treatments calculated using Student's t test. See also Figure S4.

leptin treatment increases EE when it is expressed in this way (Pelleymounter et al., 1995). Accordingly, even the data presented here would indicate hypometabolism in ob/ob mice when expressed in this way (9.8 ± 0.8 mW/g body weight in ob/ob versus 14.4 ± 0.7 mW/g in WT), as well as an effect of prolonged leptin treatment (from 12.1 ± 0.3 to 16.2 ± 0.4 mW/g body weight). However, as pointed out earlier and as previously emphasized (Butler and Kozak, 2010; Cannon and Nedergaard, 2011; Himms-Hagen, 1997; Nedergaard and Cannon, 2014; Tschöp et al., 2012), this way of normalizing the data will lead to erroneous conclusions, since it does not distinguish between metabolically active tissue and metabolically inert lipids, which account for the increased body weight seen in ob/ob mice (Figure S2I).

In contrast to the generally accepted concept of hypometabolism, our data indicate that ob/ob mice are actually hypermetabolic (see also Kaiyala et al., 2015). The hypermetabolism is not a reflection of the energetic costs of increased food intake, as the mice were not hyperphagic, nor could a larger lean mass account for the hypermetabolism of ob/ob mice, since the lean mass of ob/ob mice was not larger (Figure S2I). It may be speculated that the increased metabolism in ob/ob mice is a reflection of the extra EE needed simply to carry the increased fat

mass. However, we (Figure S2K) and others (Abreu-Vieira et al., 2015; Bray and York, 1979; Pelleymounter et al., 1995; Porter et al., 2013) found reduced locomotor activity in obese mice. Thus, it seems likely that the mice compensate for the increased cost of movement by reducing their activity. The hypermetabolism has also been interpreted to be a reflection of reduced insulation (Kaiyala et al., 2015). However, our data on tail heat loss (Figure 3), as well as the Scholander experiments (Figure 2A), do not provide any support for this hypothesis. The hypermetabolism is, therefore, not understood. The development of obesity in ob/ob mice is likely due to hyperphagia at a young age (Figure S1A) rather than to decreased thermogenesis.

Leptin-Deficient Mice Are Anapyrexic but Not Hypothermic

The reduced Tb of ob/ob mice at subthermoneutral temperatures has been interpreted as hypothermia (Bray and York, 1979; Kaiyala et al., 2015; Pelleymounter et al., 1995), which is defined as an inability to defend the Tb in the cold. However, we and others (Trayhurn and James, 1978) have found the Tb of ob/ob mice to be substantially lower than that of WT mice, but stable at subthermoneutral temperatures; particularly, we observed that the Tb did not decrease further when the degree

of cold stress was further increased (Figure 2A). This state may, therefore, be defined as being an anapyrexic state, which means that the ob/ob mice defend a Tb that is lower than the defended Tb of WT mice. However, thermal preference tests, which we (Figure 2) and others (Carlisle and Dubuc, 1984) performed to analyze behavioral thermogenesis, revealed similar temperature preference in WT and ob/ob mice. This indicates that ob/ob mice have changed thresholds of certain thermoregulatory effectors (see diagram in Figure S5). While the mice defend a lower Tb at subthermoneutral temperatures, when given the opportunity, they will behaviorally raise their Tb to WT levels.

BAT Is Not Atrophied in Leptin-Deficient Mice

The apparent hypothermia and hypometabolism of the ob/ob mice have been interpreted as being a reflection of reduced BAT non-shivering thermogenic capacity (see Introduction). In the present study, we found no evidence for reduced BAT thermogenic capacity. Although the tissue was filled with lipid, total UCP1 levels and norepinephrine responses were similar in WT and ob/ob mice (Figure 3) (Wilson et al., 1984). Therefore, despite massive lipid accumulation, the adrenergic responsiveness (Gong et al., 1997; Ricquier et al., 1986) and the recruitment state of BAT in ob/ob mice were not different from those in WT mice, nor were UCP1 mRNA levels diminished (Gong et al., 1997; Harris et al., 1998; Jacobsson et al., 1985; Ricquier et al., 1986). Furthermore, since the ob/ob mice are neither hypometabolic nor hypothermic, there is no a priori reason to expect an atrophied state of BAT, and the increased lipid accumulation is an effect of an overall high lipid load rather than of an inability of BAT to combust the lipid.

Leptin Is Not Thermogenic but Is Pyrexic

We and others observed no thermogenic response to leptin in either WT or in ob/ob mice (Döring et al., 1998; Högberg et al., 2006; Mistry et al., 1997; Rafael and Herling, 2000; Ukropec et al., 2006), at least within the accuracy of the methods used. While thermogenic effects of drugs can be masked by changes in thermoregulatory thermogenesis when administered at subthermoneutral temperatures, we were also unable to detect effects at thermoneutrality. We also found no effect in young, hyperphagic ob/ob mice (Figure S1). Norepinephrine turnover in BAT of ob/ob mice is not significantly lower than that of WT mice at thermoneutrality or in moderate cold (Zaror-Behrens and Himms-Hagen, 1983). Nonetheless, some studies reported increased BAT norepinephrine turnover in ob/ob mice due to leptin treatment (Collins et al., 1996) or increased BAT sympathetic nerve activity in rats (Morrison, 2004). We detected neither a thermogenic response nor an induction of UCP1 expression following acute or prolonged leptin treatment. There are reports, based on the results of pair-feeding experiments, that the presence of UCP1 augments the weight-diminishing effect of leptin on fat depots (body weight is not affected) (Commins et al., 2001; Okamatsu-Ogura et al., 2007). However, the extent of the repression of food intake during leptin treatment is also decreased in the absence of UCP1 (Commins et al., 2001; Okamatsu-Ogura et al., 2007, 2011). This confounds the analysis of such studies.

Although we did not observe any thermogenic effect of leptin treatment, we observed a robust increase in Tb of ob/ob mice following leptin treatment (Figures 2, 4, and S1). An increase in Tb of ob/ob mice has been reported for mice at room temperature (Pelleymounter et al., 1995; Singh et al., 2009; Ukropec et al., 2006), but, following prolonged leptin treatment, we also observed an increase in Tb at thermoneutrality (Figures 4G and 4H). Tb in WT mice also tended to be increased following leptin treatment (Figures 2 and S1). This is principally in agreement with experiments showing increased Tb in rats (De Fanti et al., 2002; Luheshi et al., 1999) or mice (Dodd et al., 2014) after leptin treatment. In the ob/ob mice, this pyrexic effect of leptin is most likely mediated by the ability to markedly decrease tail heat loss (Figure 3). Since leptin, applied in situ, has been reported to have the opposite action, i.e., a vasodilatory action, on the endothelium (Gu and Xu, 2013), the vasoconstrictory action that we observed is presumably mediated by leptin acting centrally on the sympathetic nervous system (Gu and Xu, 2013).

The way leptin alters the threshold temperature for thermoregulatory thermogenesis is not currently known. Leptin receptors are found in the preoptic chiasma anterior hypothalamus area (POAH) in the brain (Zhang et al., 2011), an area that is central for body temperature control. However, there is currently no detailed knowledge about how the thresholds for effectors of body temperature regulation are regulated, but the mechanism of leptin interaction with these processes is a challenging innovative area for understanding the function and significance of leptin.

Conclusions

For more than three decades, ob/ob mice have generally been considered to be hypometabolic and hypothermic and to have defective BAT. Subsequently, soon after its identification in 1994, leptin has been considered to be thermogenic and acting partly through BAT (re)activation. The data presented here seriously challenge these concepts. Whereas it is likely that, in a certain strain of mice—the Aston strain—the ob/ob genotype is associated with BAT atrophy (Ashwell et al., 1985; Thurlby and Trayhurn, 1980; Trayhurn and James, 1978; Wilson et al., 1984), this is clearly not a general effect of this genotype, and obesity can clearly develop in the ob/ob mice without the addition of the effect of an increased metabolic efficiency caused by the absence of UCP1 activity. Similarly, leptin can, at least in mice, clearly decrease obesity without the contribution of extra thermogenesis from BAT. Metabolically, the present studies should, therefore, help in directing further efforts concerning leptin action into its effects on the mechanisms of body temperature control.

EXPERIMENTAL PROCEDURES

Please see the Supplemental Experimental Procedures for additional details. All experiments were approved by the Animal Ethics Committee of the North Stockholm Region.

Animals

Routinely, male 12- to 24-week-old C57BL/6 Lep[ob]/JRj mice and lean littermates (Janvier Labs) were used in this study. As these mice result from heterozygote crossings, the lean controls used in this study were either +/+ or +/ob; both these genotypes are referred to as WT. The mice were housed in single cages at 21°C, with free access to water and the chow diet R70 (Lactamin) on a 12-hr/12-hr light/dark cycle. In the injection experiments, the mice were

routinely either injected with sterile saline or 5 mg/kg body weight recombinant murine leptin (PeproTech). Indirect calorimetry measurements of non-anesthetized mice were either performed at 21°C or 30°C on a 12-hr/12-hr light/dark cycle. For measurement of Tb and locomotor activity in three dimensions, the animals were implanted intraperitoneally with telemetric transponders. Tail temperatures were measured at room temperature, using a FLIR T335 infrared camera as described by Hoefig et al. (2015).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.041.

AUTHOR CONTRIBUTIONS

A.W.F., B.C., and J.N. designed the study, were involved in all aspects of the experiments, and co-wrote the manuscript. G.A.-V., J.M.A.d.J., and N.P. were responsible for the metabolic studies and BAT protein analysis. C.H. and J.M. were responsible for infrared thermography. All authors discussed the results and commented on the manuscript.

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