

A superactive leptin antagonist alters metabolism and locomotion in high-leptin mice

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Abstract

Transgenic alpha murine urokinase-type plasminogen activator (α MUPA) mice are resistant to obesity and their locomotor activity is altered. As these mice have high leptin levels, our objective was to test whether leptin is responsible for these characteristics. α MUPA, their genetic background control (FVB/N), and C57BL mice were injected s.c. every other day with 20 mg/kg pegylated superactive mouse leptin antagonist (PEG-SMLA) for 6 weeks. We tested the effect of PEG-SMLA on body weight, locomotion, and bone health. The antagonist led to a rapid increase in body weight and subsequent insulin resistance in all treated mice. Food intake of PEG-SMLA-injected animals increased during the initial period of the experiment but then declined to a similar level to that of the control animals. Interestingly, α MUPA mice were found to have reduced bone volume (BV) than FVB/N mice, although PEG-SMLA increased bone mass in both strains. In addition, PEG-SMLA led to disrupted locomotor activity and increased corticosterone levels in C57BL but decreased levels in α MUPA or FVB/N mice. These results suggest that leptin is responsible for the lean phenotype and reduced BV in α MUPA mice; leptin affects corticosterone levels in mice in a strain-specific manner; and leptin alters locomotor activity, a behavior determined by the central circadian clock.

Key Words

- ▶ α MUPA
- ▶ PEG-SMLA
- ▶ leptin
- ▶ antagonist
- ▶ bone
- ▶ locomotor
- ▶ obesity

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Introduction

Alpha murine urokinase-type plasminogen activator (α MUPA) mice carry as a transgene the cDNA-encoding uPA linked to the enhancer–promoter region of the α A-crystalline gene (Miskin *et al.* 1990). uPA is an extracellular serine protease that plays a role in fibrinolysis and tissue remodeling (Mondino & Blasi 2004) and has been implicated in brain development, synaptic plasticity, and neuroprotection (Del Bigio *et al.* 1999, Cho *et al.* 2012). α MUPA mice show transgenic expression in the ocular lens, as expected from the promoter specificity, however also ectopic expression specifically in the brain and developing teeth (Miskin *et al.* 2005, 2006). α MUPA

mice display a set of phenotypic behavioral and metabolic alterations, such as reduced food intake and body weight, resistance to obesity, increased life span, reduced incidence of cancerous lesions, and reduced serum levels of insulin-like growth factor 1 (IGF1; Miskin *et al.* 2005, Tirosh *et al.* 2005). In addition, these mice have increased serum levels of the anorexigenic hormones insulin and leptin and low serum levels of the orexigenic hormone ghrelin, along with increased transcript levels of anorexigenic neuropeptides and decreased levels of orexigenic neuropeptides in the brain (Froy *et al.* 2011). α MUPA mice also show pronounced circadian rhythms (Froy *et al.* 2006)

and altered locomotor activity compared with FVB/N mice (Gutman *et al.* 2011).

α MUPA mice that spontaneously consume less food per day (~25%) have many of the aforementioned health-related features of mice treated with caloric restriction (CR; Speakman & Mitchell 2011). However, CR-treated animals differ from α MUPA mice as they have low serum levels of leptin and insulin and high levels of ghrelin (Speakman & Mitchell 2011). Previous studies have shown that CR during rapid skeletal growth is deleterious to both cortical and trabecular bone mass and architecture (Devlin *et al.* 2010). In mice between the ages of 2.5 and 8.5 months, CR decreases femur cortical bone mass but maintains or increases trabecular bone mass, effects thought to be linked to the low leptin levels (Hamrick *et al.* 2008). Likewise, CR decreases tibiae trabecular bone mass until 1 year of age mainly through a leptin-mediated suppression of bone formation, whereas CR for more than 2 years has a protective effect against age-related bone loss through reducing the rate of bone turnover (Tatsumi *et al.* 2008). The effect of high leptin levels on bone health has never been studied in α MUPA mice.

We hypothesize that some of the aforementioned differences between α MUPA and FVB/N mice could be mediated by high α MUPA leptin levels, in particular, the satiated, lean phenotype, resistance to obesity, and the alterations in locomotor activity. As leptin is involved in bone metabolism (Hamrick 2004, Karsenty & Oury 2010), we hypothesize that α MUPA mice could also differ in bone properties from FVB/N mice. Therefore, our aim was to examine the involvement of leptin in these aspects of the α MUPA phenotype. We used a recently developed pegylated superactive mouse leptin antagonist (PEG-SMLA; Shpilman *et al.* 2011), and compared α MUPA mice, their FVB/N control, and C57BL mice that are more commonly used in metabolic studies.

Materials and methods

Animal treatments

Five-month-old α MUPA ($n=7-9$) and FVB/N (wild-type (WT)) mice ($n=7-9$) (obtained from the Weizmann Institute of Science, Rehovot, Israel) and C57BL mice ($n=8$; Harlan, Jerusalem, Israel) were housed in a facility with controlled temperature (20–22 °C) and humidity (60%) under a 12 h light:12 h darkness (LD) cycle with regular chow available *ad libitum*. After 2 weeks of acclimation, mice were injected s.c. every other day with

20 mg/kg per day PEG-SMLA for 6 weeks, 2 weeks in LD, 2 weeks in total darkness (DD), and 2 weeks in LD. PEG-SMLA was expressed, purified, and monopegylated as was described previously (Shpilman *et al.* 2011). Food intake and body weight were monitored throughout the experiment. On the last day of the experiment, mice were fasted for 10 h. Subsequently, mice were anesthetized with isoflurane and blood was collected. At the time of blood collection, fasting blood glucose levels were determined using a glucometer (Optium Xceed; Abbott Laboratories). Total fat content was determined by weighing abdominal fat pads. The Joint Ethics Committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an AAALAC International accredited institute.

Homeostasis model assessment of insulin resistance

The insulin-resistance index from fasting serum insulin and plasma glucose levels was determined by the homeostasis model assessment of insulin resistance (HOMA-IR) parameter: $\text{HOMA} = \text{fasting serum insulin } (\mu\text{U/ml}) \times \text{fasting plasma glucose (mg/dl)} / 405$.

Serum separation and ELISA

Blood was kept at room temperature for 30 min for clotting and consequently centrifuged at 2000 g for 15 min at 4 °C. Serum was collected and stored at –20 °C for further analysis. Serum hormone levels were determined for insulin (Mercodia, Uppsala, Sweden), corticosterone (Assaypro, St Charles, MO, USA), IL6, and TNF α (R&D Systems, Inc., Minneapolis, MN, USA) using ELISA Kits. Assays were performed according to the manufacturers' instructions.

Animal locomotor activity

General cage activity was monitored continuously at 6-min intervals using a custom-made system composed of infrared detectors placed above each cage, as was previously described (Gutman *et al.* 2011). Cage locomotor activity was recorded continuously under LD conditions for 14 days after which mice were released to DD for 14 days and then again to LD for 14 days. Double-plotted actograms were generated using Actogram Software. Period length of circadian activity rhythms in DD (τ) was calculated individually by χ^2 analyses using Tau Software. Actogram and Tau Softwares were kindly provided by Refinetti R, University of South Carolina.

Micro-CT analysis of third lumbar vertebrae

Third lumbar vertebrae (LV3) were scanned with a Skyscan 1174 X-ray computed microtomograph scanner (Skyscan, Aartselaar, Belgium), equipped with a Charge-Couple Device (CCD) detector. Images were obtained by 50 kV X-ray tube voltages and 800 mA current, 0.25 mm aluminum filter, at 4300 ms exposure time, and 11.1 pixel size resolution were used. For each specimen, a series of 900 projection images were obtained with a rotation step of 0.4°, averaging two frames, for a total 360° rotation. Flat field correction was performed at the beginning of each scan for a specific zoom and image format. A stack of 2D X-ray shadow projections was reconstructed to obtain images using NRecon Software (Skyscan) and subjected to the morphometric analysis using CTAn Software (Skyscan). During reconstruction, dynamic image range, post-alignment value, beam hardening, and ring-artifact reduction were optimized. For analysis of the Lumbar vertebrae LV3 trabecular region, a total of 150 slices, corresponding to 1.665 mm were selected and adaptive gray-scale threshold levels between 60 and 255 were used. Analysis of the lumbar vertebrae included the entire region of the vertebrae body. Trabecular bone measurements in the lumbar vertebra included trabecular bone volume (BV)/tissue volume (TV) (%), trabecular number (Tb.N; 1/μm), trabecular thickness (Tb.Th; μm), and trabecular separation (Tb.Sp; μm). Morphometric analysis was based on the 2D and 3D internal CTAn plug-ins. 3D images (CTM file format) were constructed from cortical and trabecular regions of interest, using Marching Cubes 33 algorithm in CTVol Software (Skyscan).

Statistical analysis

Student's *t*-test was used for comparison within mouse strains. All results are expressed as means ± s.e.m. For all analyses, the significance level was set at $P < 0.05$. Statistical analysis was performed with JMP Software (version 5.1; SAS Institute, Inc., Cary, NC, USA).

Results

We examined the impact of a PEG-SMLA on weight gain, bone health, and locomotor activity in naturally lean α MUPA mice, which express high leptin levels. α MUPA mice were compared to FVB/N mice, their WT genetic background, and to C57BL mice. All three strains were compared to their corresponding saline-injected mice. To test the effect on locomotor activity, the effect of

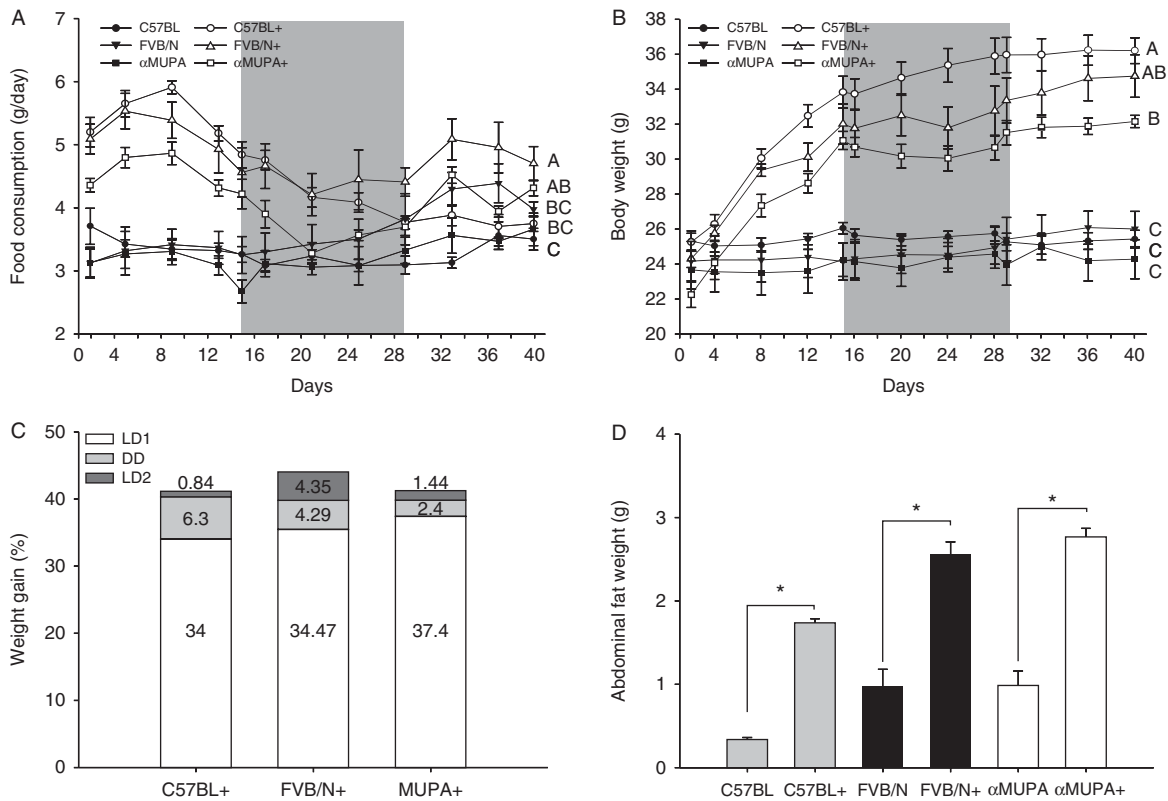
PEG-SMLA was performed for 6 weeks, 2 weeks in light–dark (LD1), 2 weeks in total darkness (DD), and then 2 weeks in LD (LD2).

Effect of PEG-SMLA on body weight and food intake

PEG-SMLA led to immediate increased food intake (Fig. 1A) and body weight (Fig. 1B) in α MUPA, FVB/N, and C57BL compared with saline-injected mice. Although α MUPA mice started with a typical lower body weight compared with WT FVB/N mice of the same age, their weight gain during the first LD period (LD1) was higher than that of FVB/N or C57BL mice (~37% compared to ~34%; Fig. 1C). Corresponding to their increased body weight, all strains showed increased abdominal fat pads, with α MUPA showing the highest (Fig. 1D), as expected from the initial increase during the LD1 period. The main weight gain was achieved during the first 2 weeks and continued, but at a lower pace, throughout the last 4 weeks. In parallel, food intake was much higher during the first week in all three strains and started to decline thereafter resembling that of the control mice (Fig. 1A). After 6 weeks, the amount of food intake was only slightly higher in the PEG-SMLA- vs saline-injected mice in the three strains, although major differences in body weight were maintained (Fig. 1A and B).

Effect of PEG-SMLA on serum parameters

We next examined serum parameters to characterize the weight gain status of the PEG-SMLA-injected mice. Glucose levels were higher in the PEG-SMLA- vs saline-injected mice, showing glucose intolerance (Fig. 2A). Also, insulin levels were higher in the PEG-SMLA-injected mice (Fig. 2B) yielding, together with the high glucose levels, a high insulin resistance index (HOMA-IR; Fig. 2C). We next analyzed whether mice in this experimental treatment were normal (glucose and insulin are <1.96 s.d. above control average), insulin-resistant (insulin level is higher than 1.96 s.d. above control average, but glucose is <1.96 s.d. above control average), or have developed type 2 diabetes mellitus (both insulin and glucose are higher than 1.96 s.d. above control average). This analysis showed that in all three strains, almost all mice injected with PEG-SMLA had above the critical level of insulin (Supplementary Fig. 1A, B and C, see section on supplementary data given at the end of this article). Determination of glucose showed that one out of seven and four out of seven of the mice injected with PEG-SMLA were slightly above the corresponding glucose critical level

**Figure 1**

Body weight, weight gain, food consumption, and fat depots of C57BL, α MUPA, and FVB/N mice injected with PEG-SMLA or saline. (A) Food consumption. (B) Weekly mean body weight. (C) Weight gain (%).

(D) Abdominal fat tissue mass. Data are means \pm s.e.m.; $n=7-9$ /group; asterisks denote significant difference ($P<0.05$); + designates PEG-SMLA-injected mice. The gray bar represents the DD period.

in α MUPA and FVB/N mice respectively while almost all C57BL mice were slightly above the critical level (Supplementary Fig. 1D, E and F). No correlation was found between the weight gain and the insulin level in all three mouse strains treated with PEG-SMLA (not shown). Analysis of two obesity pro-inflammatory markers, IL6 and TNF α , revealed no increased blood levels in all three strains (not shown). However, corticosterone levels were approximately sixfold higher in PEG-SMLA-injected C57BL mice, but lower, approximately sixfold or two-to threefold fold in α MUPA or FVB/N mice respectively (Fig. 2D).

Effect of PEG-SMLA on locomotor activity

We next compared the effect of PEG-SMLA on daily locomotor activity during LD1 and LD2. PEG-SMLA caused reduced activity in both periods (Fig. 3). To test the effect on the period of the circadian clock, mice were placed under DD condition. Analysis of the period (τ) revealed no significant difference as a result of

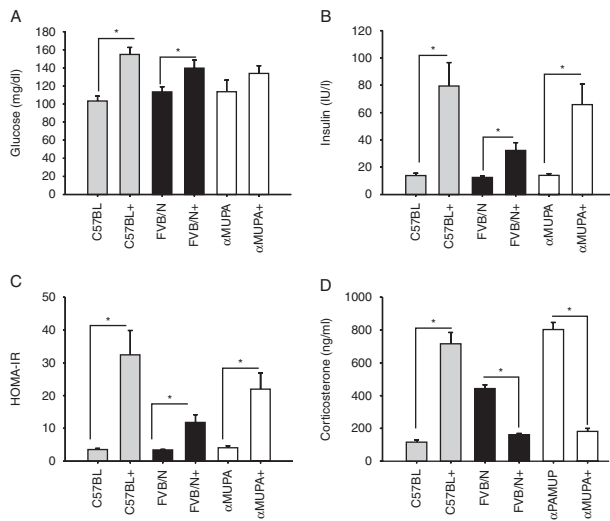
PEG-SMLA injection (Supplementary Fig. 2, see section on supplementary data given at the end of this article).

Effect of PEG-SMLA on bone health

As leptin inhibits bone accrual and α MUPA have high leptin levels, we measured the effect of PEG-SMLA on bone health. Interestingly, the BV/TV and Tb.Sp were lower in α MUPA mice compared with their WT FVB/N mice even in the saline-injected mice (Table 1). As a result of PEG-SMLA treatment, the thickness of a single trabecula (Tb.Th) did not change, but in most cases, the number of trabeculae per tissue area (Tb.N) increased. Owing to this increase, the BV/TV, which is the most important parameter in bone health, also increased, whereas the Tb.Sp consequently decreased (Table 1).

Discussion

In this study, we took advantage of a recently developed leptin antagonist, PEG-SMLA (Shpilman *et al.* 2011), to

**Figure 2**

Serum glucose and hormone levels of C57BL, α MUPA, and FVB/N mice injected with PEG-SMLA or saline. (A) Glucose. (B) Insulin. (C) HOMA-IR. (D) Corticosterone. Data are means \pm s.e.m.; $n = 7$ – 9 /group; asterisks denote significant difference ($P < 0.05$); + designates PEG-SMLA-injected mice.

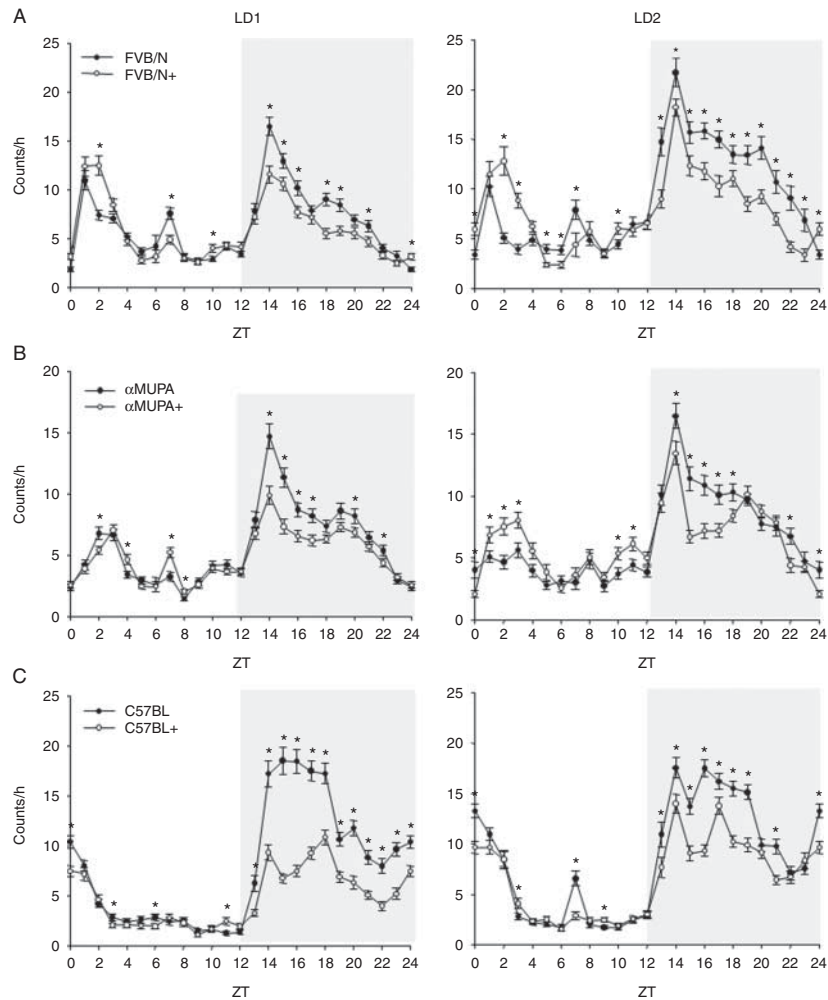
examine the extent in which leptin is responsible for several phenotypic features exhibited by α MUPA mice that express high levels of this adipokine. We found that a superactive leptin antagonist leads to fast increased body weight. In addition, we also found that it leads to disrupted locomotor activity and increased corticosterone secretion in C57BL mice but not in α MUPA or FVB/N mice.

PEG-SMLA has recently been shown to lead to $\sim 40\%$ increase in body weight (Shpilman *et al.* 2011). However, PEG-SMLA injection led to weight gain in α MUPA mice, shown previously to have higher leptin levels than their genetic background (Froy *et al.* 2011) and to be resistant to obesity throughout their lifetime (Froy & Miskin 2010). Weight gain induction in α MUPA mice suggests that these mice are lean due to the high leptin levels. The results also suggest that α MUPA mice are protected against leptin resistance, a state that appears under chronic increase in leptin (Halaas *et al.* 1997), such as during obesity (Montague *et al.* 1997). Interestingly, FVB/N mice, which have been used as a diet-induced obesity-resistant model (Kim *et al.* 2012), also became obese, suggesting that these mice are not resistant to leptin antagonist-induced weight gain. Surprisingly, we found that the weight gain seen in all mice was associated with increased food intake only during the first week of PEG-SMLA injection, but decreased to levels only slightly higher than those consumed by saline-injected mice. These results can be explained by the fact that initial

PEG-SMLA injection led to increased hunger and that, in turn, led to increased food intake. However, the secondary effect of PEG-SMLA may have been to relieve the inhibition of corticosterone secretion mediated by leptin (Malendowicz *et al.* 2007). In C57BL mice, the high levels of corticosterone could have led to increased differentiation to fat tissue (Madsen *et al.* 2005), which led to elevated body weight without increased food intake. However, in α MUPA and FVB/N mice, suppressed corticosterone secretion was seen as a result of PEG-SMLA injection. Our results suggest that primary and secondary effects on corticosterone secretion after PEG-SMLA injection in mice are strain specific and reminiscent of the findings in rats (Hochol *et al.* 2000, Malendowicz *et al.* 2007). Determination of 1.96 s.d. insulin and glucose levels suggested that all PEG-SMLA-treated mice have developed insulin resistance after 6 weeks of injection. However, only one out of seven α MUPA and four out of seven FVB/N mice, but almost all C57BL mice, were marginally diabetic. It should also be noted that the PEG-SMLA treatment that led to insulin resistance was not accompanied by inflammation in contrast with mice kept on high-fat diet (Sherman *et al.* 2011).

In all three strains, we found that PEG-SMLA injection led to decreased locomotor activity. As the circadian clock in the hypothalamus suprachiasmatic nuclei (SCN) controls rhythms of locomotor activity and this brain area has been shown to have leptin receptors (Guan *et al.* 1997, Yi *et al.* 2006), our results suggest that a leptin antagonist may counteract the effect of leptin on the SCN clock. Indeed, it has recently been shown that leptin potentiated the phase-shifting effect of a 30-min light pulse on behavioral rhythms during the late subjective night in female mice (Mendoza *et al.* 2011). In contrast to our results, it was found that a 2-week chronic exposure to a physiological dose of leptin (100 μ g/kg per day) decreased locomotor activity (Mendoza *et al.* 2011). As obesity itself has been shown to also affect locomotor activity (Kohsaka *et al.* 2007, Sherman *et al.* 2011), it is possible that leptin resistance, a characteristic of obesity, is the reason for the disrupted locomotor activity.

Our results show that α MUPA mice have a more reduced BV than their WT genetic background. α MUPA display several characteristics common to CR-treated animals, such as reduced food intake, increased longevity, reduced body temperature, reduced levels of serum IGF1, increased levels of mitochondrion-mediated apoptosis, and reduced incidence of spontaneous and induced cancerous lesions (Tirosh *et al.* 2003, 2005, Miskin *et al.* 2005). However, the reduced BV found in α MUPA mice are inconsistent with CR-treated mice,

**Figure 3**

Locomotor activity of C57BL6, α MUPA, and FVB/N mice injected with PEG-SMLA or saline. (A) Mean locomotor activity of FVB/N mice. (B) Mean locomotor activity of α MUPA mice. (C) Mean locomotor activity of C57BL mice. Results are mean activity during the last 14 days of LD1 or LD2 period.

Values are means \pm s.e.m.; $n=7-9$ /group; shaded area designates the darkness phase; asterisks denote significant difference ($P<0.05$); + designates PEG-SMLA-injected mice.

as previous studies show that CR preserves or increases trabecular BV (Hamrick *et al.* 2008) and retards age-related bone loss in aged animals (Pendergrass *et al.* 1995, Tatsumi *et al.* 2008). As low levels of leptin lead to improved bone

health (Hamrick 2004, Karsenty & Oury 2010), the high leptin levels in α MUPA mice may account for their reduced BV. This also corroborates our findings that the PEG-SMLA injection led to significantly increased BV in FVB/N mice and

Table 1 Bone parameters of saline- vs PEG-SMLA-injected α MUPA and FVB/N mice. Results are mean \pm s.e.m. ($n=7-9$)

	Saline-injected FVB/N	PEG-SMLA-injected FVB/N	Saline-injected α MUPA	PEG-SMLA-injected α MUPA
BV/TV (%)	15.6 \pm 1.07 ^b	19.75 \pm 1.15 ^a	11.9 \pm 0.64 ^c	13.3 \pm 0.86 ^{b,c}
Tb.Th (μ m)	0.07 \pm 0.001 ^a	0.07 \pm 0.0008 ^a	0.07 \pm 0.001 ^a	0.07 \pm 0.00 ^a
Tb.Sp (μ m)	0.34 \pm 0.01 ^{b,c}	0.30 \pm 0.01 ^c	0.44 \pm 0.02 ^a	0.40 \pm 0.02 ^{a,b}
Tb.N (1/ μ m)	2.15 \pm 0.13 ^{a,b}	2.62 \pm 0.14 ^a	1.64 \pm 0.09 ^b	1.76 \pm 0.11 ^b

Results marked with the same letter are not statistically different.

to a higher although not significant BV/TV in α MUPA mice. These results are also corroborated by other findings that show increased bone formation in C57BL mice after PEG-SMLA treatment (Solomon, Gertler, and Ornan-Monsonego, unpublished results). In addition, the elevated levels of corticosterone in α MUPA mice could also account for the reduced BV.

In summary, after applying a powerful leptin antagonist, we conclude that the endogenous high leptin levels found in α MUPA mice are responsible to a large extent for their reduced food intake, lean phenotype, resistance to obesity, and weak bones. Also, the effect of the antagonist, and therefore of leptin, on corticosterone serum levels is strain specific in mice. As the leptin antagonist leads to decreased locomotor activity in all three mouse strains tested, our results also support a leptin action on the SCN clock. Thus, a high-leptin model with an antagonist that induces weight gain without having to use high-fat diet may be used in future studies to decipher the effect of leptin on metabolism, bone health, and the circadian clock.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0033>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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