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Muscle-specific interleukin-6 deletion influences body weight and body fat in a sex-dependent manner

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ABSTRACT

Interleukin-6 (IL-6) is a major cytokine controlling not only the immune system but also basic physiological variables such as body weight and metabolism. While central IL-6 is clearly implicated in the latter, the putative role of peripheral IL-6 controlling body weight remains unclear. We herewith report results obtained in muscle-specific IL-6 KO (*mIL-6 KO*) mice. *mIL-6 KO* male mice fed a high-fat diet (HFD, 58.4% kcal from fat) or a control diet (18%) gained less weight and body fat than littermate floxed male mice, while the opposite pattern was observed in female mice. Food intake was not affected by muscle IL-6 deficiency, but male and female *mIL-6 KO* mice were more and less active, respectively, in the hole-board test. Moreover, female *mIL-6 KO* mice did not control adequately their body temperature upon exposure to 4 °C, suggesting a role of muscle IL-6 in energy expenditure. At least part of this regulatory role of muscle IL-6 may be mediated by the hypothalamus, as IL-6 deficiency regulated the expression of critical hypothalamic neuropeptides (NPY, AgRP, POMC, CRH and preproOX). Leptin and insulin changes cannot explain the phenotype of these mice. In summary, the present results demonstrate that muscle IL-6 controls body weight and body fat in a sex-specific fashion, influencing the expression of the main neuropeptides involved in energy homeostasis.

1. Introduction

Obesity and type 2 diabetes mellitus (T2DM) are a major worldwide health problem (Kasuga, 2006; Ogawa and Kasuga, 2008). It is becoming increasingly recognized that obesity results in chronic low-grade inflammation caused by dysregulation of cytokines such as interleukin-6 (IL-6) (Fain et al., 2004; Kasuga, 2006; Wellen and Hotamisligil, 2005). The concept of adipokines, hormone-like factors produced by adipose tissue and overproduced by expanded fat during obesity, was an exciting novel idea that has attracted notable interest. Thus, circulating IL-6 levels strongly correlate with adipose tissue mass (Bastard et al., 2000; Fain et al., 2004; Mohamed-Ali et al., 1997; Vozarova et al., 2001). IL-6 is also a myokine, a factor expressed by skeletal muscle. Furthemore, it is upregulated by exercise and is secreted into the bloodstream in response to muscle contractions (Steensberg et al., 2000), probably working as an energy sensor in the muscle (Febbraio et al., 2003) as well as controlling muscle mass (Serrano et al., 2008). IL-6, a cytokine originally identified as a B-cell differentiation factor (BSF-2) into antibody-producing cells, is the founder member of the neuropoietins and therefore is also secretable by many immune and neural cells (Erta et al., 2012; Hirano et al., 1985; Kishimoto et al., 1995).

A seminal study in Jansson's lab (Wallenius et al., 2002b) demonstrated that total IL-6 KO mice develop mature-onset obesity, strongly suggesting that IL-6 is involved in the control of body weight; in addition, these authors reported that IL-6 deficiency induced altered leptin levels and insulin sensitivity. Evidence also showed that IL-6 acting at the central level may be essential for the role of this cytokine as a regulator of energy homeostasis. Thus, a single i.c.v. injection of IL-6 acutely increases energy expenditure (Wallenius et al., 2002b). Chronic i.c.v. administration of IL-6 (Wallenius et al., 2002a), adeno-associated viral delivery of IL-6 in rat hypothalamus (Li et al., 2002) or central

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nervous system-restricted over-expression of IL-6 (Hidalgo et al., 2010; Señarís et al., 2011) decreased weight gain and fat content. Moreover, we recently demonstrated that conditional IL-6 KO mice where IL-6 expression is ablated specifically in astrocytes also show mature-onset obesity (Quintana et al., 2013). It is likely that IL-6 causes these effects through dysregulation of the main hypothalamic neuropeptides involved in energy homeostasis (Benrick et al., 2009; Schéle et al., 2013, 2012, Señarís et al., 2011).

Circulating IL-6 eventually may have access to the CNS (Pan and Kastin, 2007) and regulate the energy balance, but may also affect peripheral tissues. Increased lipolysis and fat oxidation, hepatic glucose production, muscle glucose uptake and regulation of other cytokines and hormones are among the functions assigned to IL-6 in this regard. Whether peripheral IL-6 is involved in body weight regulation, remains to be firmly established. To give some insight into this problem, we have produced floxed mice for IL-6 (Quintana et al., 2013) and deleted its expression specifically in muscle. Food intake and body growth were monitored, whereas putative energy expenditure was evaluated by examining the response to cold exposure and by looking at the general activity of the animals in the Hole-Board, a test that allows to separate locomotion from exploration (Boissier and Simon, 1962; File and Wardill, 1975) and is preferable to the open field test, which poorly discriminates spontaneous locomotor activity, exploration and anxiety (Archer, 1973). Critical hypothalamic factors and hormones as well as physiological responses to glucose and insulin challenges were also evaluated. The present study demonstrates a significant role of muscle IL-6 in body weight and fat regulation in a sex-dependent manner, likely acting, at least in part, through trough hypothalamic pathways.

2. Materials and methods

2.1. Animals

To produce muscle-specific IL-6 KO mice and proper controls, LoxP-flanked (floxed) mice for the *Il-6* gene, recently generated in our laboratory (Quintana et al., 2013) were first crossed with heterozygous *myosin light chain 1f* (*Mlc*)-*Cre* mice, provided by Dr. Steve Burden (Bothe et al., 2000). The offspring positive for Cre were selected and crossed with the floxed mice (*Il-6*$^{lox/lox}$) again to obtain the genotypes to be studied: *Mlc-Cre*$^{+/-}$ *Il-6*$^{lox/lox}$, which lack IL6 expression in skeletal muscle cells; *Mlc-Cre*$^{+/-}$ *Il-6*$^{lox/+}$ which will be heterozygous for the conditional deletion in muscle; *Mlc-Cre*$^{-/-}$ *Il-6*$^{lox/lox}$; and *Mlc-Cre*$^{-/-}$ *Il-6*$^{lox/+}$. For the sake of simplicity, we will call these groups *muscle-Il6 KO*, heterozygous, floxed and wild-type mice, respectively. The mice were genotyped by PCR analyses using tail genomic DNA.

All mice were kept under constant temperature and under a standard 12-h light/12-h dark cycle and with free access to food and water unless otherwise stated. Several experiments were carried out as outlined in Fig. 1A. Ethical approval for the use of all mice in this study was obtained from the Autonomous University of Barcelona Animal Care and Ethics Committee.

2.2. Diets

Diets were obtained from Harlan Iberica: the high-fat diet (HFD) TD 03584 (58.4% kcal from fat), and the control diet 2018 (18% kcal from fat). Mice were put on the HFD when they were ~1 month old for 12–14 weeks in two separate diet experiments (n = 8–22 and 10–19); Fig. 1A (top) outlines one of them. Body weight and food intake were monitored weekly and 2–3 times a week, respectively. In most cases mice were not isolated but kept housed after weaning 2–5 per cage according to their genotype and sex. Animals from these experiments were tested in the hole-board in week 10 of diet, and subjected to an insulin tolerance test (ITT) and an oral glucose tolerance test (OGTT) in weeks 10 and 11 of HFD, respectively, as outlined in Fig. 1A (top). Mice were killed by decapitation one week later, the blood collected from the trunk and allowed to clot for obtaining the serum, where several hormones and metabolites were subsequently analyzed. The brains were carefully removed and frozen in dry ice for further use for *in situ* hybridization analysis of several neuropeptides. Liver, visceral (gonadal) and subcutaneous (dorsal area) fat depots and brown adipose tissue were removed and weighed upon euthanasia of the animals, and either fixed in 4% paraformaldehyde or snap-frozen in liquid nitrogen for further analysis (RT-qPCR, adipocyte size and number, liver steatosis). Tibialis and gastrocnemius muscles and tibia were also obtained; the latter was cleaned up for measuring its length and calculate tibialis/tibia ratios.

2.3. Adipocyte size and number

Gonadal and subcutaneous adipose tissues of ~16 week-old floxed and *muscle-Il-6 KO* mice fed control and HFD (n = 10–19) were fixed in 4% paraformaldehyde for 24 h and subsequently paraffin-embedded. Eight-micrometer sections were cut and stained with haematoxylin & eosin. They were examined with a Nikon Eclipse E400 microscope interfaced to a DXM 1200 camera. Pictures were taken at 40× magnification. The area of at least 300 adipocytes for each animal was measured using ImageJ (Abramoff et al., 2004) developed by U.S. National Institutes of Health (http://rsb.info.nih.gov/ij/). To determine the fat cell number we used the procedure described by Lemonnier (Lemonnier, 1972). Briefly, adipocyte volume (V) was calculated from the mean adipocyte surface area (S) by de formula $V = 4 \cdot S^{3/2}/(3 \cdot \sqrt{\pi})$. Fat cell number was estimated from the ratio, adipose tissue weight/mean adipose cell volume × 0.92 (density of the adipose tissue).

2.4. Histological analysis of liver steatosis

Hepatic steatosis was assessed by oil red-O (ORO) staining in OCT-embedded cryosections of ~16 week-old floxed and *muscle-Il-6 KO* mice fed control and HFD (n = 9–19). They were examined with a Nikon Eclipse 90i microscope interfaced to a DXM 1200F camera. Pictures were taken at 20× magnifications using the software ACT-1 version 2.70 (Nikon corporation). A minimum of 10 independent fields per sample was evaluated. Relative areas of steatosis (expressed as percent oil red-O staining) were quantified using image analysis software ImageJ.

2.5. Behavioral tests

The locomotion and exploratory behaviours of ~14 week-old floxed and *muscle-Il-6 KO* mice fed control diet (n = 10–12) were evaluated with the hole-board apparatus, which consists of a white conglomerate wood box (40 × 40 × 20 cm) with four 3-cm diameter equidistant holes on the floor. The floor is divided in 16 squares of 10 × 10 cm marked with black lines, conforming 12 external squares (around the walls) and 4 internal squares. A video camera was placed to allow recording of the whole session. Hole-board test area lighting was approximately 90 lux. Mice were analyzed as follows. On test day, mice were taken to the behavior room at ~2 PM and remained there for 20 min. Prior to placing the animal in the hole-board, the box was wiped with a 5% ethanol solution and dried. Mice were placed in the middle of the box and tested for 5 min. Frequency and duration of head-dipping events (considered to be when an animal inserted the head into the holes at least to its ear level) were recorded *in situ* while the number of crossings of the squares (horizontal activity) and rearings (vertical activity)