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Increased leptin and white adipose tissue hypoplasia are sexually dimorphic in *Lif* null/*Igf-I* haploinsufficient mice

Carmen Fernández-Moreno[a], Jose G. Pichel[b], Vera Chesnokova[c], Flora De Pablo[a,*]

[a] *Group of Growth Factors in Vertebrate Development, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (C.S.I.C.), Ramiro de Maetzu 9, 28040 Madrid, Spain*
[b] *Centro de Investigación del Cáncer, Consejo Superior de Investigaciones Científicas (C.S.I.C.), Salamanca, Spain*
[c] *Cedars Sinai Research Institute-UCLA School of Medicine, Los Angeles, CA, USA*

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Abstract We previously showed cooperation of leukemia inhibitory factor (LIF) and insulin-like growth factor I (IGF-I) during development. Mice doubly deficient in LIF and IGF-I died at birth. We now analyze the possible combined influence of both factors on postnatal growth. The haploinsufficiency of the *Igf-I* gene on a *Lif* null background caused a marked reduction in body mass index and white adipose tissue only in female mice. These animals had increased leptin, increased serum IGF-I and apparent substitution of white adipose tissue by brown adipose tissue. The complex interrelationships between LIF and IGF-I in regulating weight thus involve sexually dimorphic effects on adipose tissue differentiation and circulating leptin.

Key words: Insulin-like growth factor; Leukemia inhibitory factor; Leptin; Adipose tissue

1. Introduction

Leukemia inhibitory factor (LIF) and insulin-like growth factor I (IGF-I) are members of two protein signaling families with a broad spectrum of common cellular targets [1,2]. In adult mice, LIF absence causes a 30% weight reduction [3], whereas IGF-I absence causes a 70% decrease in growth [4]. In a previous study, we demonstrated cooperation between LIF and IGF-I to achieve normal fetal growth as well as lung, bone and muscle development. Indeed, mice doubly deficient in LIF and IGF-I are growth-retarded and do not survive after birth [5]. Little is known, however, of the physiological cooperation of LIF and IGF-I in vivo in postnatal life.

LIF effects on cell proliferation and differentiation are mediated by a dimeric receptor of the cytokine family that also includes the growth hormone (GH) and leptin receptors [6]. Multiple cellular functions are influenced by IGF-I through a well known tyrosine kinase receptor of the insulin receptor family [2]. Evidence has emerged of cross-talk between the signal transduction pathways of cytokine receptors and insulin/IGF-I receptors at several levels [7,8].

Leptin, a hormone secreted mainly by white adipose tissue (WAT) and also by brown adipose tissue (BAT) [9], is another major regulator of body weight and energy balance. It was recently reported that leptin and insulin combine additively to reduce food intake and body weight in rats [10]. The goal of the present study was to analyze in vivo the combined impact of LIF absence ($L^{-/-}$) and IGF-I haploinsufficiency ($I^{+/-}$) on postnatal growth and on adipose tissue physiology, to gain insight into the complexity of sexually dimorphic body weight control.

2. Materials and methods

2.1. Mouse production, maintenance and growth

The mutant mice for *Igf-I* and *Lif* genes were kindly provided by Drs. Argiris Efstratiadis (Columbia University, NY, USA) and Colin L. Stewart (National Cancer Institute, Frederick, MD, USA), respectively [3,4]. The generation and genotyping of the double mutant mice has been described [5]. The original mice were bred on a mixed 129/sv×MF1×C57Bl6/j background. Mice were maintained following established animal care recommendations at the Centro de Investigaciones Biológicas on a 12 h light:dark cycle, were fed ad libitum and were weaned at 3 weeks. Mice were weighed weekly from postnatal weeks 3 to 12. Body length (nose to anus) was measured at 8 weeks and body mass index (BMI, weight/length^2) was calculated.

2.2. Serum biochemical parameters and statistical methods

Blood was collected without anesthesia from the orbital plexus, obtained after overnight fasting. Serum was stored at −20°C. An enzymatic assay was used to determine total triacylglycerol (Sigma, St. Louis, MO, USA). Glucose was measured with a glucometer using blood glucose strips (Roche Diagnostics, Mannheim, Germany). Serum IGF-I (DSL, Webster, TX, USA) and leptin (Linco, St. Charles, MO, USA) were measured by radioimmunoassay. Statistical analysis was performed first with a one-way ANOVA for multiple comparisons, applying Tukey's correction and then a Student's *t*-test for comparison of the more significant phenotypes.

2.3. Northern blot

Total pituitary RNA was extracted with Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Northern analysis of pituitary GH expression was performed as described [11,12] using 20 μg total pituitary RNA/lane. The GH probe is a 700 kb *Nde*I fragment of human growth hormone 1 gene (ATCC, Rockville, MA, USA), the β-actin probe (Mouse DECAprobe template, Ambion, Austin, TX, USA) is a 1.076 kb fragment of the mouse cytoplasmic β-actin gene. Probes were labeled by random priming with RadPrime DNA Labeling System (Invitrogen).

2.4. Histology

Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated in ethanol, and paraffin-embedded. Blocks were sectioned at 7 μm and stained with hematoxylin/eosin (H&E) or with antiserum raised in rabbit (kind gift of Dr. Eduardo Rial, CIB, CSIC [13]) against uncoupling protein 1 (UCP1).

*Corresponding author. Fax: (34)-91-534 9201.
E-mail address: fdepablo@cib.csic.es (F. De Pablo).

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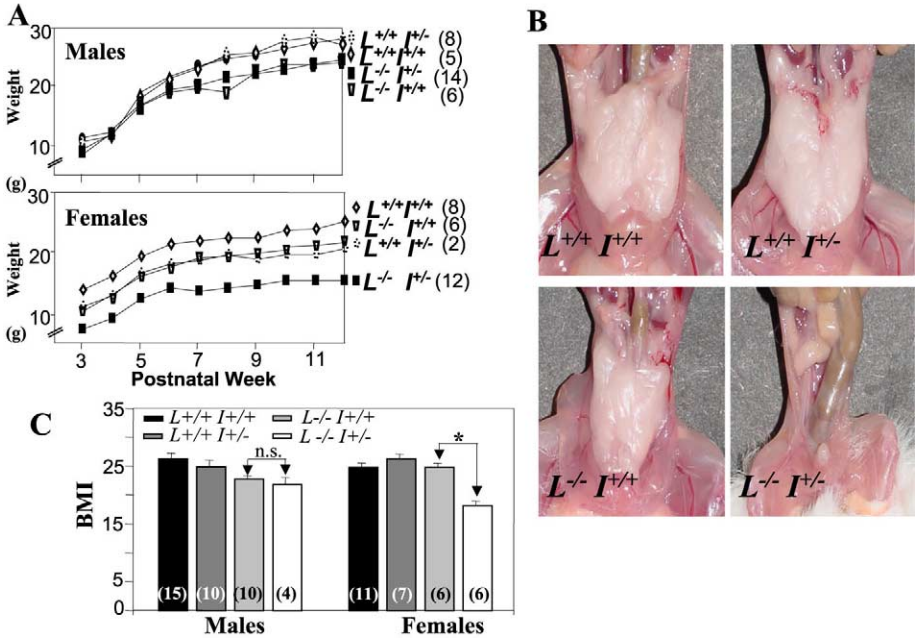

Fig. 1. Postnatal growth curves and phenotypes of adult mice. A: Body weight from weeks 3 to 12, in males and females. Note the arrested weight gain in the $L^{-/-}I^{+/-}$ females after 6 weeks. Genotypes and number of animals (in parentheses) are shown. B: Perigonadal adipose tissue of 12 week old female mice. C: BMI of 8 week old mice. $^{*}P < 0.05$, by Student's *t*-test.

3. Results

3.1. Sexually dimorphic postnatal growth retardation

Mutant animal growth curves revealed important differences between genotypes. Body length was similar regardless of *Lif* null mutation or *Igf-I* haploinsufficiency; only *Igf-I* null mice were, as expected, dwarf (data not shown). Weight, in contrast, was always lower in the $L^{-/-}I^{+/-}$ females than the rest of animals studied, and weight gain was essentially arrested in them after 6 weeks (Fig. 1A). These females were thin with marked reduction of perigonadal WAT (Fig. 1A,B), but appeared otherwise normal; at least some had an estrous cycle based on cell characteristics of vaginal smears. At 8 weeks, their BMI was lower ($P < 0.05$) than that of the $L^{-/-}I^{+/+}$ females (Fig. 1C). The growth curve in male $L^{-/-}I^{+/-}$ mice was indistinguishable from that of *Lif* null and wild type males up to 6 weeks.

3.2. Metabolic parameters and pituitary GH expression

There were no significant differences in fasting glucose levels at 8 weeks of age in any group. Triglycerides were high in the *Lif* null females, but the values were normal in the $L^{-/-}I^{+/-}$ females. Serum IGF-I concentrations, unexpectedly, were higher than normal in male and female $L^{-/-}I^{+/+}$ as well as $L^{-/-}I^{+/-}$ mice, although this was statistically significant only in females (Table 1). Both the $L^{+/-}I^{+/-}$ and $L^{-/-}I^{+/-}$ mice had slightly increased pituitary GH expression (Fig. 2).

3.3. Increased serum leptin

Compared to wild type mice, leptin tended to increase in IGF-I haploinsufficient or *Lif* null animals, in both males and females, but increased synergistically only in $L^{-/-}I^{+/-}$ females (3.9 ng/ml in the $L^{-/-}I^{+/-}$ females vs. 2.8 ng/ml in $L^{-/-}I^{+/-}$ males as well as in $L^{-/-}I^{+/+}$ females) (Fig. 3).

3.4. Decreased WAT in double mutant females

Macroscopic analysis of the perigonadal WAT deposits showed a marked reduction in $L^{-/-}I^{+/-}$ females (Fig. 1B), whereas the interscapular BAT deposits were apparently normal (data not shown). Histologically, the WAT adipocytes were depleted of lipid droplets and the tissue presented a very compact morphology (Fig. 4), similar to the BAT of wild type animals. The BAT substituting normal WAT in mutant females had high expression of UCP1 (Fig. 5). In

Table 1
Comparison of metabolic parameters at 8 weeks

Genotype	Females				Males			
Lif Igf-I	+/+ +/+	+/+ +/−	−/− +/+	−/− +/−	+/+ +/+	+/+ +/−	−/− +/+	−/− +/−
Glucose	63.1 ± 6.7	57.5 ± 8.7	49.2 ± 4.1	56.2 ± 6.7	47.7 ± 10.7	49.4 ± 6.1	44.08 ± 5.5	57.2 ± 7.5
(*n*)	(14)	(13)	(9)	(14)	(8)	(11)	(12)	(9)
Triglycerides	53.9 ± 6.7	60.6 ± 2.3	82.1 ± 7.2*	60 ± 3.1	85.4 ± 12.2	77.4 ± 6.7	65.02 ± 2.9	55.8 ± 1.9
(*n*)	(7)	(7)	(7)	(7)	(7)	(5)	(6)	(3)
IGF-I	478 ± 58.8	470 ± 53.4	832.1 ± 113*	718.7 ± 57.7*	506.11 ± 35.5	426.2 ± 32.5	642.1 ± 58.7	655.5 ± 84.9
(*n*)	(10)	(5)	(7)	(16)	(9)	(4)	(7)	(10)

Glucose and triglyceride concentrations are given in mg/dl and IGF-I concentration is in ng/ml.
Mean ± S.E.M. is shown. A one-way ANOVA test was performed initially; $^{*}P < 0.05$, by Student's *t*-test with respect to wild type animals.

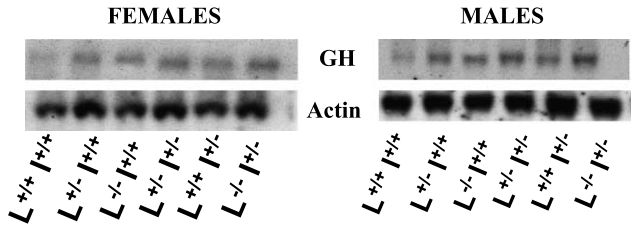

Fig. 2. Northern blot analysis of pituitary GH mRNA ($n = 6$–8 animals per group). 20 μg/lane total RNA was analyzed. A representative blot is shown. Relative abundance of GH mRNA was determined by densitometry, normalized to levels of β-actin mRNA in the same sample. In females, $I^{+/-}L^{+/-}$ demonstrated a 34% increase and $I^{+/-}L^{-/-}$ a 25% increase of GH mRNA as compared to wild type mice. In males, $I^{+/-}L^{+/-}$ showed a 44% increase and $I^{+/-}L^{-/-}$ a 55% increase as compared to wild type males.

$L^{-/-}L^{+/+}$ males there was a partial substitution of the WAT by BAT, although surprisingly this phenotype was largely rescued in the $L^{-/-}I^{+/-}$ males (Figs. 4 and 5).

4. Discussion

The network of factors influencing adipose tissue differentiation, metabolism and body mass in mammals is complex and sexually dimorphic. The multifunctional, highly glycosylated cytokine LIF is unusual in this context, as both LIF overexpression [14] and LIF deficiency [3] cause decreased body weight. It therefore appears critical for adequate weight control to maintain normal LIF levels, which act in endocrine and paracrine modes in the periphery, as well as in the central nervous system [1,10]. Indeed, central LIF gene therapy in rats has been shown to suppress food intake, body weight and induce a decrease in leptin and insulin [15]. The less understood decreased growth caused by the absence of LIF [3] is confirmed in the present study, which we show is due to a decrease in BMI and not to decreased skeletal growth. This absence of longitudinal growth retardation concurs with the finding that LIF deficiency tended to increase serum IGF-I, even in *Igf-I* haploinsufficient mice. This was particularly marked in $L^{-/-}I^{+/-}$ females, with IGF-I values significantly above those of wild type mice. Since the mutant mice had increased pituitary GH mRNA expression, our data support that LIF deficiency causes abnormally high function of the GH–IGF-I axis, which overcompensates the *Igf-I* haploinsufficiency. This is concordant with the profound influence of LIF in pituitary development. When LIF was overexpressed locally in pituitary in transgenic mice, it induced a Cushingoid syndrome with somatotroph and gonadothroph hypoplasia and low plasma IGF-I [16]. Leptin levels were not reported in these mice.

The phenotype observed in the adipose tissue therefore appears to be a result of LIF deficiency in the presence of slightly higher than normal circulating IGF-I in the $L^{-/-}I^{+/-}$ mice. Whether the local adipose tissue IGF-I concentration is low or high is presently unknown. As reflected by the weight gain curves, the phenotype in our study was markedly influenced by the sex of the mice. Young adult $L^{-/-}I^{+/-}$ female mice are significantly smaller than the males of the same genotype, whose growth curves are very similar to the *Lif* null mice. Interestingly, it has been demonstrated that mice deficient in liver production of IGF-I display sexual dimorphism in GH-stimulated postnatal growth, and this effect was not provoked by gonadal steroids [17].

The fact that preadipocytes secrete LIF and that an antagonist of the LIF receptor inhibits adipogenesis in vitro indicates that, in addition to central effects on body weight, LIF influences adipocyte differentiation directly [18]. It was thus not surprising to find a slight decrease in lipid droplets in the

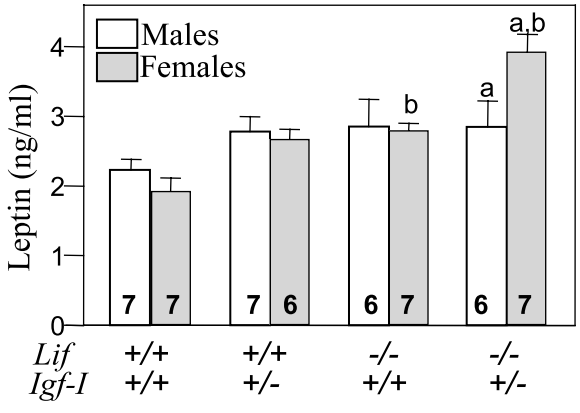

Fig. 3. Serum leptin in 8 week old male and female mice. The number of animals is indicated. a and b are comparisons with the equivalent letter; $P < 0.05$, by Student's *t*-test. *Lif* null females, but not males, had significantly higher values than their wild type controls.

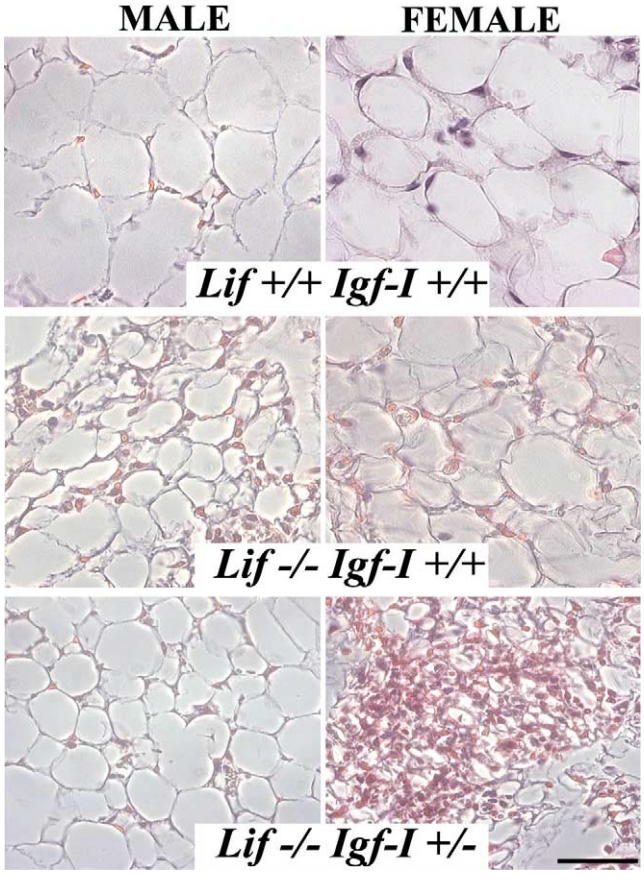

Fig. 4. Perigonadal WAT histopathology in male and female mice. H&E-stained, representative cross-sections are shown. Genotypes indicated apply to each row. Bar, 40 μm.

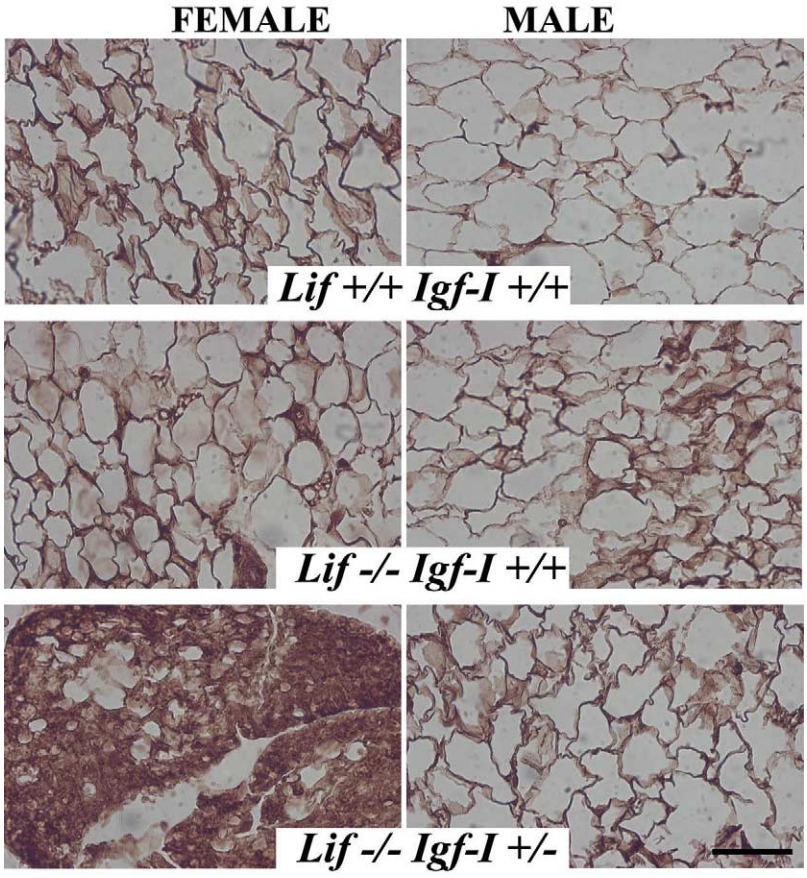

Fig. 5. UCP1 expression in representative sections of perigonadal WAT in female and male mice. Genotypes indicated apply to each row. Bar, 40 μm.

WAT of both male and female $L^{-/-}I^{+/+}$ mice. The apparent substitution of WAT by BAT, only in the $L^{-/-}I^{+/-}$ females, was nonetheless unpredicted. This recruitment appears to be due to the LIF deficit which induces increased leptin, further increased in the *LIF* null *Igf-I* haploinsufficient female mice. Indeed, leptin has been shown to inhibit marrow stromal cell differentiation to adipocytes [19] and to increase lipolysis in WAT and BAT [20].

In recent years, the role of leptin has been extended to many more effects than that of an adipostatic, anti-obesity hormone [21]. The source of leptin in the WAT-deficient mutant mice remains undetermined. Either the increased BAT deposits [9] or the pancreas [22] may synthesize it. A peripheral mechanism of the sexual dimorphism in leptin secretion by WAT has been reported [23] but no information is available relative to BAT. While our study leaves many questions open, it emphasizes that a combinatorial approach must be taken to unravel the complex physiology of adipose tissue as a sexually dimorphic endocrine tissue. Novel LIF and IGF-I central and local interrelationships, different in both sexes, appear to be important modulators of leptin control and the fine equilibrium of body weight.

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