Original Article

MOLECULAR METABOLISM

Suppressing hyperinsulinemia prevents obesity but causes rapid onset of diabetes in leptin-deficient *Lep*$^{ob/ob}$ mice

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ABSTRACT

Objective: Hyperinsulinemia is commonly associated with obesity. Mice deficient in the adipose-derived hormone leptin (*Lep*$^{ob/ob}$) develop hyperinsulinemia prior to onset of obesity and glucose intolerance. Whether the excess of circulating insulin is a major contributor to obesity and impaired glucose homeostasis in *Lep*$^{ob/ob}$ mice is unclear. It has been reported previously that diet-induced obesity in mice can be prevented by reducing insulin gene dosage. In the present study, we examined the effects of genetic insulin reduction in *Lep*$^{ob/ob}$ mice on circulating insulin, body composition, and glucose homeostasis.

Methods: Leptin expressing (*Lep*$^{wt/wt}$) mice lacking 3 insulin alleles were crossed with *Lep*$^{ob/ob}$ mice to generate *Lep*$^{ob/ob}$ and *Lep*$^{wt/wt}$ littermates lacking 1 (*Ins1*$^{+/+}$;*Ins2*$^{+/-}$), 2 (*Ins1*$^{+/+}$;*Ins2*$^{-/-}$) or 3 (*Ins1*$^{+/-}$;*Ins2*$^{-/-}$) insulin alleles. Animals were assessed for body weight gain, body composition, glucose homeostasis, and islet morphology.

Results: We found that in young *Lep*$^{ob/ob}$ mice, loss of 2 or 3 insulin alleles reduced plasma insulin levels by 75–95% and attenuated body weight gain by 50–90% compared to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice. This corresponded with ~30% and ~50% reduced total body fat in *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice, respectively. Loss of 2 or 3 insulin alleles in young *Lep*$^{ob/ob}$ mice resulted in onset of fasting hyperglycemia by 4 weeks of age, exacerbated glucose intolerance, and abnormal islet morphology. In contrast, loss of 1,2 or 3 insulin alleles in *Lep*$^{wt/wt}$ mice did not significantly alter plasma insulin levels, body weight, fat mass, fasting glycemia, or glucose tolerance.

Conclusion: Taken together, our findings indicate that hyperinsulinemia is required for excess adiposity in *Lep*$^{ob/ob}$ mice and sufficient insulin production is necessary to maintain euglycemia in the absence of leptin.

Keywords Hyperinsulinemia; *Lep*$^{ob/ob}$; Obesity; Hyperglycemia

1. INTRODUCTION

Obesity has a significant impact on human health yet the mechanisms underlying it are poorly understood. Mutation of the gene encoding leptin in mice (*Lep*$^{ob/ob}$) results in a deficiency of leptin, and increases in hyperphagia and adiposity [1]. In addition, loss of leptin also impairs glucose homeostasis, manifesting as glucose intolerance, and, at times, fasting hyperglycemia in mice greater than 8 weeks of age [2]. In both *Lep*$^{ob/ob}$ and mice lacking leptin receptors (*Lepr*$^{db/db}$), hyperinsulinemia occurs prior to increases in body weight and fasting glucose levels [2–4], possibly as a result of removal of leptin's inhibitory effects on insulin gene expression and secretion [5,6]. Administration of exogenous leptin to *Lep*$^{ob/ob}$ mice results in attenuation of circulating insulin levels and improved fasting glycemia prior to reductions in body weight [7]. Conversely, insulin upregulates the production of leptin [8]. The opposing actions of insulin and leptin results in an adipoinsular feedback loop that plays an important role in regulating fat deposition.

The sequence of events resulting in increased adiposity, hyperinsulinemia, and insulin resistance that occur in obesity is not fully understood. In human studies, elevated insulin levels predict obesity later in life [9], suggesting that hyperinsulinemia contributes to excess adiposity. In obese rodents, suppression of insulin secretion using diazoxide lowers body weight [10,11]. However, diazoxide can act independently of reducing insulin levels to attenuate adiposity and food intake [12]. Thus while these studies suggest that lowering insulin pharmacologically may be a successful strategy to reduce body weight, the confounding effects of diazoxide [10,13] make it difficult to delineate the direct effects of insulin on adiposity. To understand the direct implications of circulating insulin levels on obesity, an alternative strategy involves direct modification of insulin gene expression. In rodents, two insulin genes exist, *Ins1* and *Ins2*. Previous studies demonstrated that reduction of plasma insulin levels

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by lowering insulin gene dosage to either a single *Ins1* or *Ins2* allele protected mice against high fat diet-induced obesity [14,15]. To determine whether hyperinsulinemia plays a causal role in metabolic defects including obesity and glucose intolerance that develop in the absence of leptin, we assessed the effects of reduced insulin gene dosage in $Lep^{ob/ob}$ mice. Our results indicate that reducing insulin gene dosage attenuated hyperinsulinemia and prevented obesity in $Lep^{ob/ob}$ mice. However, curtailing hyperinsulinemia in $Lep^{ob/ob}$ mice also resulted in the development of hyperglycemia. Therefore, in the absence of leptin, sufficient insulin levels are required to prevent diabetes.

2. METHODS

2.1. Experimental animals

Leptin-treated (mouse recombinant leptin, Peprotech, Rocky Hill, NJ) $Lep^{ob/ob}$ C57Bl/6J mice were crossed with $Ins1^{+/-}$;$Ins2^{-/-}$:$Lep^{wt/wt}$ mice (mixed genetic background, predominately C57BL/6J and 129 strains) to generate mice heterozygous for a mutation in the gene encoding leptin ($Lep^{ob/wt}$). Offspring from these mice were then bred with one another to generate male and female $Lep^{ob/ob}$ mice and $Lep^{wt/wt}$ mice lacking 2 ($Ins1^{+/+}$;$Ins2^{-/-}$) or 3 ($Ins1^{+/-}$;$Ins2^{-/-}$) copies of the insulin gene. Because it was not possible to efficiently generate $Lep^{ob/ob}$ littermates with all 4 copies of insulin using the breeding strategy described above, littermates lacking 1 insulin allele ($Ins1^{+/+}$;$Ins2^{+/-}$ $Lep^{ob/ob}$) were used as experimental controls (Supplemental Figure 1). For all experiments, $Lep^{ob/ob}$ mice of varying insulin dosage were compared with leptin expressing littermates ($Lep^{wt/wt}$) of matching insulin gene dosage. Mice were housed on a 12 h light/12 h dark cycle and had *ad libitum* access to food (chow diet 2918, Harlan Laboratories, Madison WI, USA) and water. At 8 weeks of age, animals were fasted for 4 h prior to euthanasia. Body length was measured at time of euthanasia. Gonadal fat pads and pancreas were collected and weighed prior to fixation. All procedures with animals were approved by the University of British Columbia Animal Care Committee and carried out in accordance with the Canadian Council of Animal Care guidelines.

2.2. Plasma analyte & hepatic lipid measurements

Body weight, blood glucose and plasma analytes were measured following a 4-h fast. Blood glucose was monitored via a One Touch Ultra Glucometer (Life Scan, Burnaby, Canada) from the saphenous vein. Insulin (Mouse Ultrasensitive Insulin ELISA, Alpco Diagnostics, Salem, NH, USA) was measured from plasma collected at 5 or 7 weeks of age. Circulating glucagon (Glucagon ELISA, Millipore, St Charles, MO, USA), free fatty acids (HR Series NEFA Kit; Wako Chemicals, Richmond, VA, USA), and triglycerides (Serum triglyceride kit; Sigma–Aldrich St. Louis, MO, USA) were measured from plasma obtained from blood collected via cardiac puncture at 8 weeks of age. At 7 weeks of age, mice underwent a 6 h fast prior to oral gavage of dextrose (1.5 g/kg, 40% solution) to assess glucose tolerance and glucose stimulated insulin secretion. At time = 0, 15, 30, 60, and 120 min, blood glucose was measured to assess glucose tolerance and plasma was collected to assess circulating insulin levels. Hepatic triglycerides were extracted as previously described [16] by a protocol modified from Briaud et al. [17] and measured using a Serum Triglyceride Determination kit (Serum triglyceride kit; Sigma–Aldrich St. Louis, MO, USA).

2.3. Metabolic analysis

PhenoMaster metabolic cages (TSE Systems, Chesterfield MO, USA) were used for measure of activity, food intake, RER, and energy expenditure (indirect calorimetry) in male mice. At 5 weeks of age, male mice were housed individually and allowed to acclimate in training chambers for 4 days prior to placement in metabolic cages. The cages were placed in environmental control chambers at 21 °C for measurement of oxygen and carbon dioxide gas exchange, physical activity (beam breaks) and food intake over 72 h; data from the first 24 h were discarded. Data from two full light and dark cycles were averaged [18]. Body fat mass and lean mass were measured using dual X-ray absorptiometry (DEXA, Lunar PIXImus densitometer, GE Medical Systems LUNAR, Madison, WI, USA).

2.4. Pancreas immunohistochemistry and morphology

Pancreata were harvested from male mice at 8 weeks of age, post-fixed overnight in 4% paraformaldehyde, rinsed in 70% ethanol, and embedded in paraffin. Paraffin sections (5 μm) were prepared by Wax-it Histology Services (Vancouver, Canada). Immunofluorescence staining was performed as previously described [19]. Pancreas sections were immunostained for insulin (rabbit anti-insulin antibody, 1:1000; C27C9, Cell Signaling Danvers MA, USA) and glucagon (mouse anti-glucagon antibody, 1:1000; G2654, Sigma–Aldrich, St. Louis MO, USA) and counterstained with DAPI to identify nuclei. Total insulin-positive area or glucagon-positive area was expressed relative to whole pancreas area and averaged across three pancreas sections per mouse, as previously described [20].

2.5. Statistical analysis

Statistical analysis was performed using Prism 6.0 software with a P value < 0.05 indicative of significance. All values are expressed as mean ± SEM unless otherwise indicated. A one way ANOVA, two-way ANOVA, or student's t-test were use as indicated, with Tukey analysis performed if significance was found. ANCOVA was used to adjust calorimetry measurements for body composition using lean mass + 0.3× fat mass as a covariate (Systat 13, San Jose CA, USA). Figures depict absolute values whereas statistics were applied to ANCOVA adjusted values [21].

3. RESULTS

3.1. Hyperinsulinemia is prevented in $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ and $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice

To determine if reduced insulin gene dosage is sufficient to protect $Lep^{ob/ob}$ mice from developing obesity and impaired glucose homeostasis, we generated $Lep^{ob/ob}$ mice lacking 1 ($Ins1^{+/+}$;$Ins2^{+/-}$;$Lep^{ob/ob}$), 2 ($Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{ob/ob}$) or 3 ($Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$) insulin alleles, as well as leptin-expressing mice lacking 1, 2, or 3 insulin alleles ($Ins1^{+/+}$;$Ins2^{+/-}$;$Lep^{wt/wt}$, $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{wt/wt}$, and $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{wt/wt}$, Supplementary Figure 1). We first determined the effects of reduced insulin gene dosage on plasma insulin levels. In male $Lep^{wt/wt}$ mice, insulin levels were similarly low in mice lacking 1, 2 or 3 insulin alleles at both 5 and 7 weeks of age (Figure 1A–B). In contrast, male $Ins1^{+/+}$;$Ins2^{+/-}$;$Lep^{ob/ob}$ mice were hyperinsulinemic. Loss of 2 or 3 insulin alleles in male $Lep^{ob/ob}$ mice resulted in plasma insulin levels that were comparable to $Lep^{wt/wt}$ littermates (Figure 1A–B). Female $Lep^{ob/ob}$ mice lacking 3 insulin alleles also had comparable plasma insulin levels to $Lep^{wt/wt}$ littermates, whereas plasma insulin levels of female $Lep^{ob/ob}$ mice lacking 2 insulin alleles were higher than $Lep^{wt/wt}$ littermates (Figure 1C–D). Despite a significant decrease of circulating insulin levels in female $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice compared to $Ins1^{+/+}$;$Ins2^{+/-}$;$Lep^{ob/ob}$ mice, insulin levels remained significantly higher than $Lep^{wt/wt}$ littermates by 7 weeks of age.

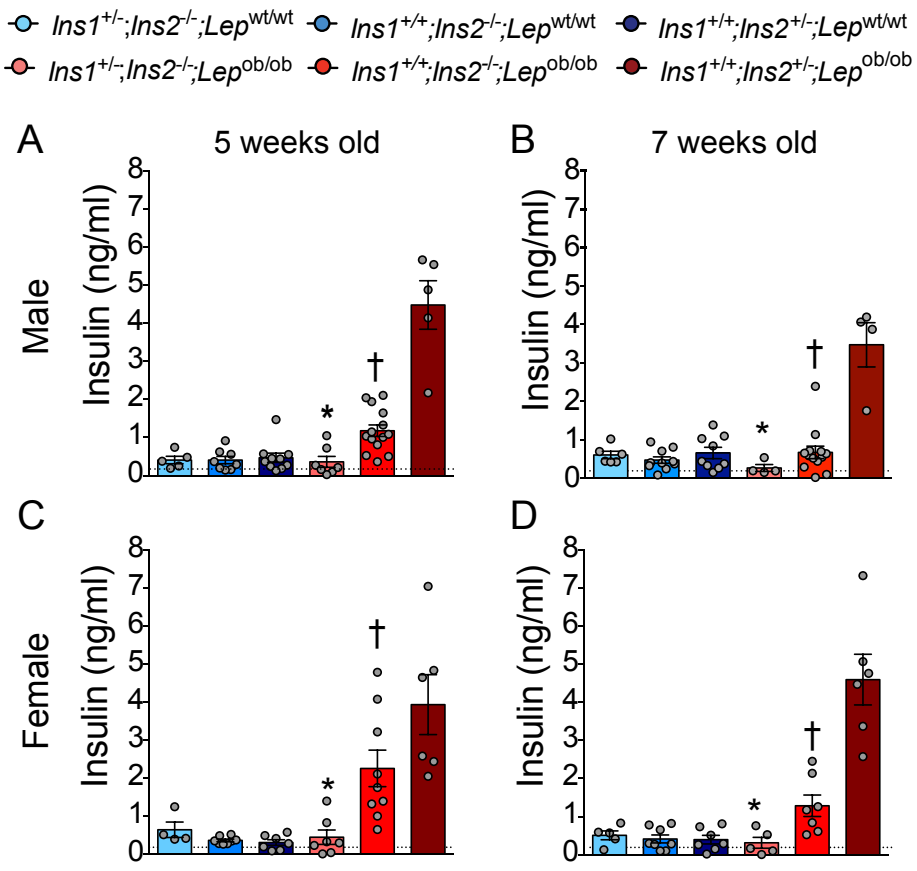

Figure 1: Insulin levels were measured following a 4 h fast in male (A, B) and female (C, D) mice at 5 and 7 weeks of age. Values are presented as mean ± SEM. A One Way ANOVA was used to assess significance. *P < 0.05 $Ins1^{+/+}$;$Ins2^{+/-}$;$Lep^{ob/ob}$ vs. $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$. †P < 0.05 $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ vs. $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$. Dotted lines on graph indicate detection limit of assay.

3.2. Reducing insulin gene dosage in $Lep^{ob/ob}$ mice protects against obesity

Since insulin is a potent anabolic hormone, we tested whether reductions of insulin gene dosage in the absence of leptin would protect against obesity. Body weight of male and female $Ins1^{+/+}$;$Ins2^{+/-}$;$Lep^{ob/ob}$ mice doubled between 4 and 8 weeks of age, and these mice were significantly heavier than all other groups (Figure 2A,B; $P < 0.05$). Body weight of $Lep^{wt/wt}$ mice did not differ between insulin gene dosage groups in both males and females. In $Lep^{ob/ob}$ mice lacking 2 or 3 insulin alleles, differences in body weight were evident as early as 5 weeks of age. Reductions in body weight reflected a similar pattern to circulating insulin levels, with lower insulin gene dosage corresponding to a greater attenuation of body weight gain. By 8 weeks of age, body weight of male $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice was comparable to $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{wt/wt}$ littermates while body weight of $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice was significantly lower than $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{wt/wt}$ littermates. In contrast, body weight of female $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice was significantly higher than $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{wt/wt}$ littermates, but body weight of $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice was comparable to $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{wt/wt}$ mice. $Lep^{wt/wt}$ and $Lep^{ob/ob}$ mice were monitored up to 8 weeks of age, beyond which point $Lep^{ob/ob}$ mice lacking 2 or 3 insulin alleles had reduced survival compared to $Lep^{wt/wt}$ littermates (Supplementary Figure 2).

The lower body weight of male $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice compared to $Lep^{wt/wt}$ mice was suggestive of growth impairments. To determine if reduced insulin gene dosage impacted growth, we assessed nasal-anus length at 8 weeks of age. While not significant, we observed a trend for reduced body length of male $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice compared to male $Ins1^{+/+}$;$Ins2^{+/-}$;$Lep^{ob/ob}$ mice (Figure 2C).

3.3. Reduction of insulin attenuates adiposity in $Ins1^{+/+}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice

To further examine the relationship between hyperinsulinemia and obesity in the context of leptin deficiency, we next examined body composition and gonadal fat pad mass. Through DEXA analysis at 5 weeks of age, the total lean and lipid mass were measured and expressed as a fat/lean ratio. Fat/lean mass of $Lep^{wt/wt}$ mice were comparable among groups lacking 1, 2 or 3 insulin alleles. As expected, fat/lean mass was highest in $Lep^{ob/ob}$ mice lacking 1 insulin allele. Reducing insulin gene dosage in male $Lep^{ob/ob}$ mice resulted in corresponding reductions of fat/lean mass (Figure 3A, Supplementary Figure 3). Loss of 2 or 3 insulin alleles resulted in a similar reduction of fat/lean mass in female $Lep^{ob/ob}$ mice compared to $Lep^{ob/ob}$ female mice lacking 1 insulin allele (Figure 3B). As reductions of fat mass are often associated with changes to lipid metabolism, we assessed plasma triglyceride and free fatty acid levels. Plasma triglyceride levels were highest in female $Ins1^{+/-}$;$Ins2^{-/-}$;$Lep^{ob/ob}$ mice (Figure 3E–F) and these mice also demonstrated a trend for the highest plasma free fatty acid levels (Figure 3C–D). To assess ectopic lipid deposition, hepatic triglyceride content was measured at 8 weeks of age. $Lep^{ob/ob}$

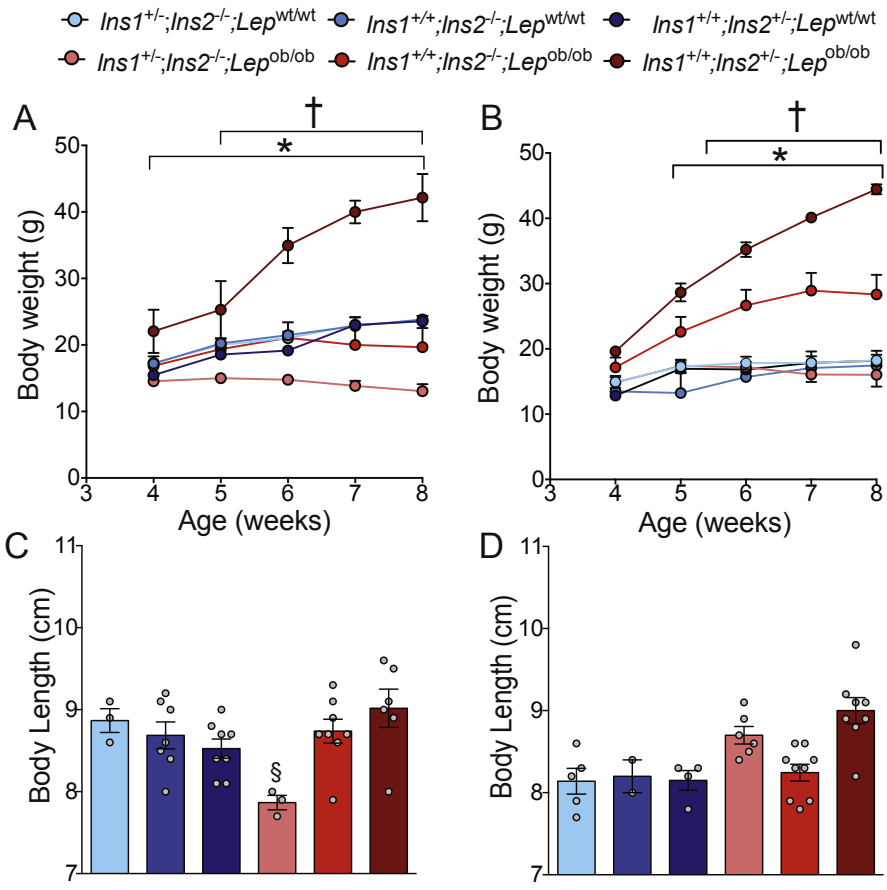

Figure 2: Body weight was measured weekly following a 4 h fast in male (A) and female (B) mice. Body length (naso-anus length) was measured at 8 weeks of age (C–D). Values are presented as mean ± SEM. A One Way ANOVA was used to assess significance. *P < 0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ vs. all other groups. †P < 0.05 *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$. §P < 0.05 *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$.

males and females lacking 1 or 2 insulin alleles had higher hepatic triglyceride content as compared to *Lep*$^{wt/wt}$ littermates (Figure 3G–H). In male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice, hepatic triglyceride levels were comparable to *Lep*$^{wt/wt}$ littermates. No significant differences in hepatic triglyceride content were observed between *Lep*$^{ob/ob}$ and *Lep*$^{wt/wt}$ females.

3.4. Attenuation of hyperinsulinemia accelerates development of hyperglycemia in *Lep*$^{ob/ob}$ mice

As insulin is essential to glucose homeostasis, we next assessed whether reduction of insulin gene dosage affects fasting blood glucose levels in *Lep*$^{ob/ob}$ mice between 4 and 8 weeks of age. *Lep*$^{wt/wt}$ mice lacking 1, 2 or 3 insulin alleles had fasting glucose levels ranging from 4 to 12 mM that did not significantly differ between insulin gene dose groups (Figure 3I,J). Blood glucose of male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice were comparable to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{wt/wt}$ mice between 4 and 7 weeks of age; however, by 8 weeks of age, *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice were hyperglycemic (blood glucose = 24.0 ± 0.35 mM). In comparison, *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ male mice were hyperglycemic (16.2 ± 5.6 mM and 21.0 ± 5.12 mM) at 4 weeks of age. *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice experienced a gradual rise in fasting glucose levels over time to 27.4 ± 2.2 mM by 8 weeks of age, while fasting glucose rose above 25 mM by 5 weeks of age in *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice and remained elevated. In contrast to the mild hyperglycemia of 4 week old male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice, fasting glucose levels of female *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice were comparable to *Lep*$^{wt/wt}$ littermates at 4 weeks of age (Figure 3J). By 8 weeks of age, blood glucose levels of *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice were elevated to >26 mM. By comparison, severe hyperglycemia (>29 mM) was evident in female *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice at 4 weeks of age and remained above 25 mM for the duration of tracking. Since hyperglycemia may also be a result of impaired alpha cell function, we next assessed plasma glucagon levels in 8-week-old male and female mice. Fasting glucagon levels did not correlate with plasma glucose levels (Figure 3K–L). Male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice had significantly lower plasma glucagon levels despite having no differences in fasting glucose levels compared to *Lep*$^{ob/ob}$ mice lacking 1 or 3 insulin alleles.

3.5. Adiposity is reduced in *Lep*$^{ob/ob}$ mice lacking 2 insulin alleles independent of changes in food intake or energy expenditure

To examine the physiological mechanisms of improved body composition in *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice, we used indirect calorimetry to assess energy expenditure and food intake at 5 weeks of age. Due to the severe hyperglycemia of male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice, comparisons of energy expenditure and food intake were only made between *Lep*$^{ob/ob}$ mice lacking 1 or 2 insulin alleles and their respective *Lep*$^{wt/wt}$ littermate controls. *Lep*$^{ob/ob}$ mice lacking 1 or 2 insulin alleles had significantly higher food intake and reduced physical activity compared to *Lep*$^{wt/wt}$ mice (Figure 4A,B). No

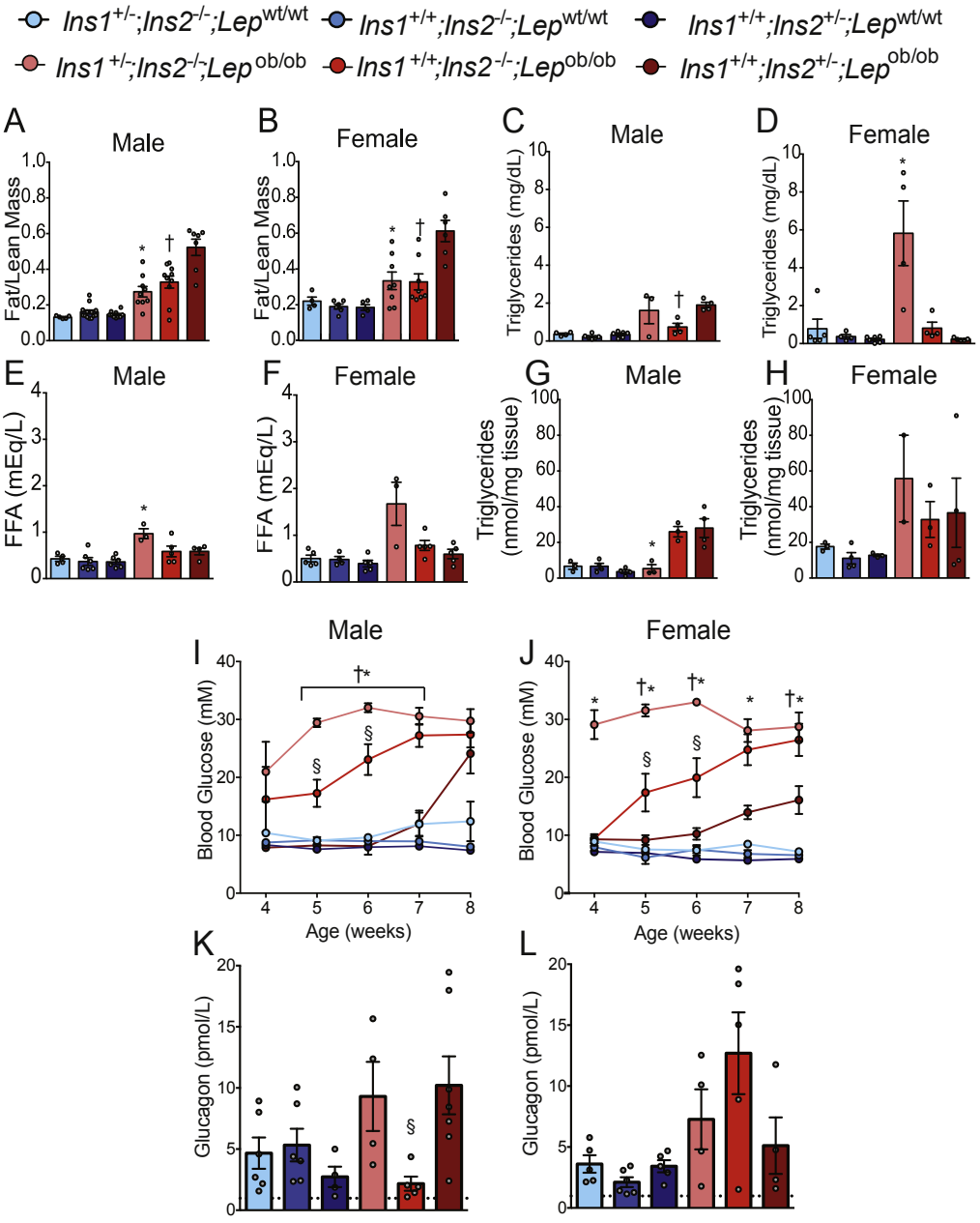

Figure 3: At 5 weeks of age, body composition was assessed using dual X-ray absorptiometry. Total body fat to lean ratio was assessed in 5 week old male (A) and female (B) mice. At 8 weeks of age, cardiac plasma was collected following euthanization for assessment of plasma triglycerides (C–D) and free fatty acids (E–F). Hepatic triglycerides were measured from livers flash frozen at 8 weeks of age (G–H). Blood glucose was measured weekly following a 4 h fast in male (I) and female (J) mice. Fasting glucagon levels were measured from plasma collected at 8 weeks of age in male (K) and female (L) mice. Values are presented as mean ± SEM. A One Way ANOVA was used to assess significance. *P $<$ 0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$; †P $<$ 0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$. §P $<$ 0.05 *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$.

differences in respiratory quotient, physical activity, or food intake occurred between male *Lep*$^{ob/ob}$ mice lacking 1 or 2 insulin alleles (Figure 4A–C). Energy expenditure was highest in male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice (heat, adjusted for lean body mass and 0.3× fat mass [21]; Figure 4D; Supplementary Figure 4C). No differences in VO_2 or VCO_2 adjusted for lean mass were observed between groups (Supplementary Figure 4). These data suggest that the improvements to body weight in *Lep*$^{ob/ob}$ mice with reduced insulin gene dosage occur independent of increases in energy expenditure at the time of these measurements.

3.6. Reduction of insulin alleles exacerbates glucose intolerance and glucose stimulated insulin secretion in *Lep*$^{ob/ob}$ but not *Lep*$^{wt/wt}$ mice

The development of hyperglycemia by 4 weeks of age in *Lep*$^{ob/ob}$ mice with reduced insulin gene dosage indicates that reduction of insulin in *Lep*$^{ob/ob}$ mice is detrimental to glucose homeostasis. To further examine the effects of reducing insulin gene dosage on glucose tolerance, we conducted an oral glucose tolerance test (GTT) and measured plasma insulin levels at selected time intervals in 7-week-old mice (Figure 5A,B). Blood glucose levels of *Lep*$^{wt/wt}$ littermates

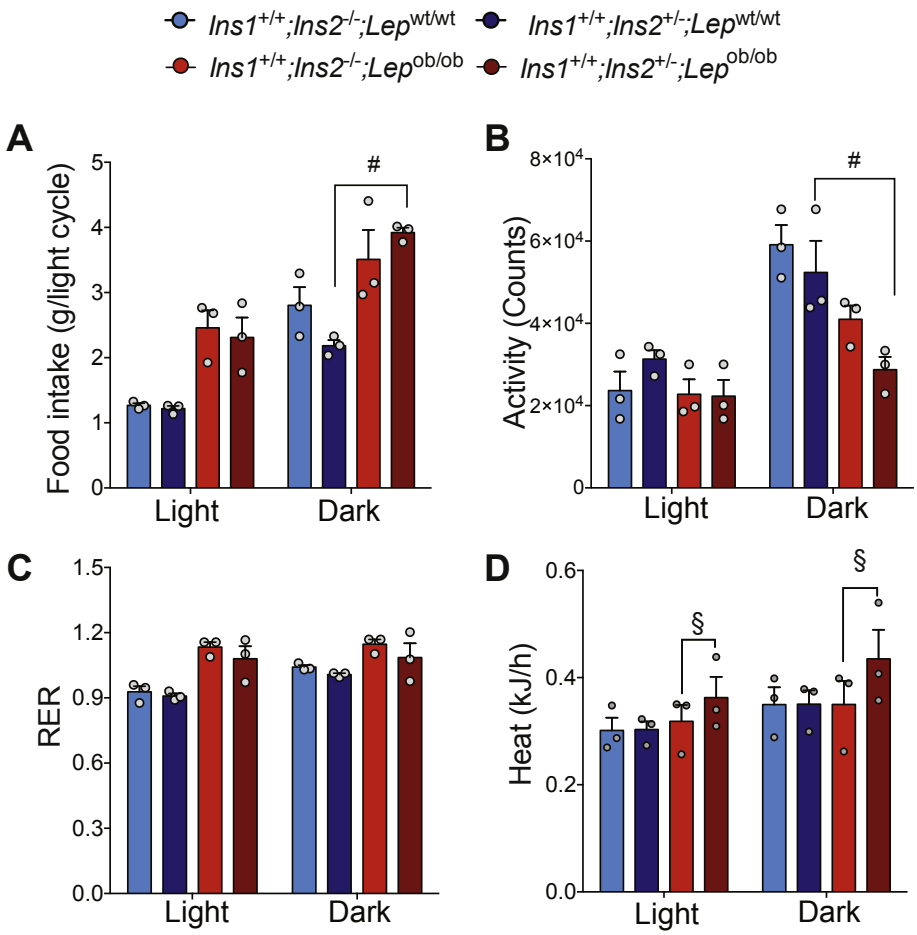

Figure 4: Food intake (A), activity (B), RER (C) and energy expenditure (D) was measured by indirect calorimetry in male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ and *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice and male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{wt/wt}$ and *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{wt/wt}$ mice at 5–6 weeks of age. Data are presented as unadjusted values. To assess statistical differences, all measures were adjusted for lean mass + 0.3× fat mass by ANCOVA followed by Tukey post hoc analysis. Values are presented as mean ± SEM. #P < 0.05 *Lep*$^{ob/ob}$ vs. *Lep*$^{wt/wt}$ groups; §P < 0.05 *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$.

lacking 1, 2 or 3 insulin alleles peaked at ~16 mM but returned to baseline levels 2 h after glucose gavage. While fasting and 2-hour post gavage glucose levels of male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice were comparable to *Lep*$^{wt/wt}$ littermates, male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice experienced a higher peak of blood glucose levels than *Lep*$^{wt/wt}$ mice. In comparison, male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice were hyperglycemic at baseline, and blood glucose rose to levels above the detection limit of the glucose meter for the duration of the GTT.

In contrast to males, female *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice had elevated blood glucose following a 6 h fast (blood glucose = 11.6 ± 1.02 mM), but trends in glucose tolerance were otherwise comparable between male and female mice. Area under the curve (AUC) analysis of glucose excursion reflected similar exacerbation of glucose intolerance among *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ and *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ male and female mice compared to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice. To determine if glucose intolerance was due to altered insulin kinetics, plasma samples collected at 0, 15, 30, and 60 min during the OGTT were assayed for insulin. Male and female *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ and *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice maintained hyperinsulinemia relative to *Lep*$^{wt/wt}$ littermates for the duration of the OGTT. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ male and female mice failed to increase insulin secretion in response to glucose and had insulin levels equal to or below *Lep*$^{wt/wt}$ (Figure 5C,D).

Expansion of beta cell area is a defining feature of *Lep*$^{ob/ob}$ mice. To determine whether reduced insulin gene dosage alters beta cell area, we co-stained pancreas sections taken from 3 different regions of the pancreas for insulin and glucagon. Islets from *Lep*$^{ob/ob}$ mice lacking 2 or 3 insulin alleles appeared distorted in shape (Figure 6A). Quantification of relative beta and alpha cell areas revealed a trend for reduced insulin positive area and increased glucagon positive area in *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice (Figure 6B,C). In contrast, islet morphology and relative beta and alpha cell area were comparable between *Lep*$^{wt/wt}$ mice lacking 2 or 3 insulin alleles.

4. DISCUSSION

In rodents lacking leptin or its receptor, hyperinsulinemia has been observed prior to onset of obesity [2]. Given recent studies in mice implicating hyperinsulinemia as a cause for obesity [14], we investigated whether the early and persistent hyperinsulinemia in *Lep*$^{ob/ob}$ mice is a major contributor to their obesity. Unlike models of high fat feeding in which leptin levels rise with increases in adiposity, the present study sought to directly determine the contribution of insulin to body weight changes in leptin deficient mice, a model of extreme obesity. Here, we report that genetic reduction of 2 or 3 insulin alleles in leptin deficient mice prevents the onset of hyperinsulinemia and corresponds with a decrease in body weight and fat/lean mass.

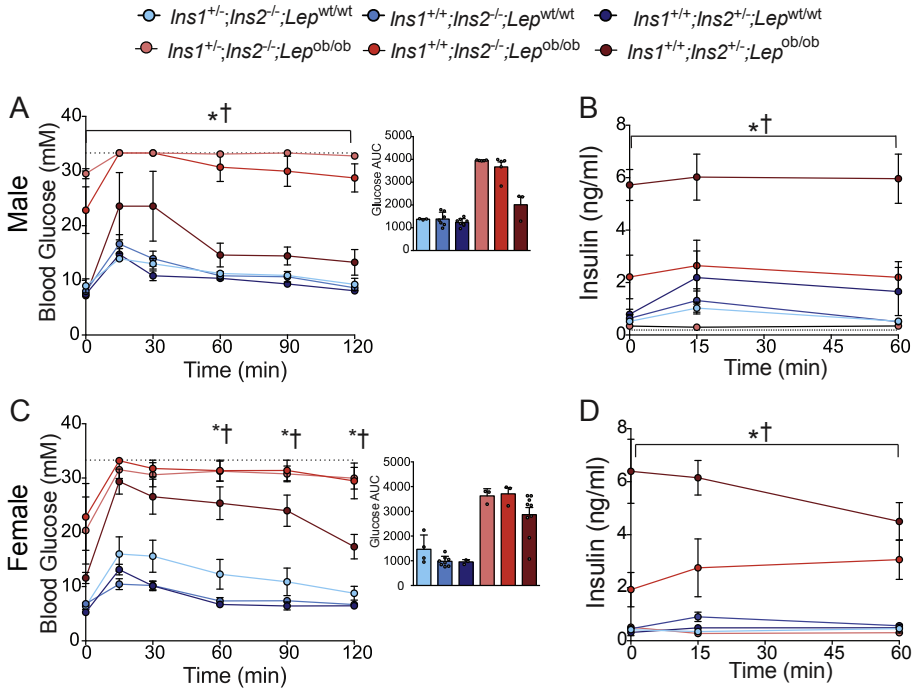

Figure 5: At 7 weeks of age, an oral glucose tolerance test was performed following a 6 h fast (A, C). Plasma was collected to measure insulin during the OGTT (B, D). Values are presented as mean ± SEM. A One Way ANOVA was used to assess significance. *P < 0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$; †P < 0.05 *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ vs. *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$.

In *Lep*$^{ob/ob}$ mice lacking 2 or 3 insulin alleles, reduced weight gain is not observed until 5 weeks of age, and is consistent with a previous study reporting no differences in body weight of pre-weaning mice lacking 2 or 3 insulin alleles [15]. Since lipogenesis is driven by insulin [22] and adipose tissue expansion occurs after weaning in rodents [23], we speculate that the attenuated weight gain observed after 4 weeks of age in *Lep*$^{ob/ob}$ mice lacking 2 or 3 insulin alleles is due, in part, to reduced insulin-stimulated adipogenesis and lipogenesis and not to developmental abnormalities.

The degree of hyperglycemia reported here in *Lep*$^{ob/ob}$ mice lacking 2 or 3 insulin alleles is greater than previously reported in high fat diet-fed *Ins1*$^{+/-}$;*Ins2*$^{-/-}$ mice [14]. Though we did not assess urine glucose output in our study, it is likely that energy lost via this route contributed to the reduced body weight and size and diminished the availability of glucose uptake into peripheral tissue such as muscle, adipose, and liver in male *Ins1*$^{+/-}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ [24]. Given the increases in energy expenditure that occurred in high fat fed mice with reduced insulin gene dosage [14], we hypothesized that reducing insulin levels may increase energy expenditure in *Lep*$^{ob/ob}$ mice. However, heat production of *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ mice was decreased compared to *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ animals, suggesting that increased energy expenditure does not mediate the decrease in body weight between male *Ins1*$^{+/+}$;*Ins2*$^{-/-}$;*Lep*$^{ob/ob}$ *and* *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice. Thus, in addition to changes in adipose metabolism, it is likely that increased urinary glucose output also contributes to the reduced body mass observed in *Lep*$^{ob/ob}$ mice lacking 2 or 3 insulin alleles, while increased energy expenditure does not appear to be a major contributor.

Interestingly, sexual dimorphism was observed in *Lep*$^{ob/ob}$ mice lacking 2 or 3 insulin alleles. In male *Lep*$^{ob/ob}$ mice, loss of 2 insulin alleles reduced insulin levels and body weight to levels comparable to *Lep*$^{wt/wt}$ littermates, and hyperglycemia was observed at 4 weeks of age. In contrast, plasma insulin levels and body weight of female *Lep*$^{ob/ob}$ mice lacking 2 insulin alleles remained higher than *Lep*$^{wt/wt}$ littermates, and onset of hyperglycemia did not occur until 5 weeks of age. In male *Lep*$^{ob/ob}$ mice, loss of 3 insulin alleles resulted in significantly lower body weight and length than in *Lep*$^{wt/wt}$ mice. This reduction in body weight and size was not observed in female *Lep*$^{ob/ob}$ mice lacking 3 insulin alleles, despite having similar fasting glucose and insulin levels. Sexual dimorphism was also observed upon assessment of plasma lipid levels and ectopic lipid accumulation. Hepatic triglyceride levels of male *Lep*$^{ob/ob}$ mice lacking 3 insulin alleles were comparable to *Lep*$^{wt/wt}$ mice. In contrast, female *Lep*$^{ob/ob}$ mice lacking 2 or 3 insulin alleles had higher plasma lipid and hepatic triglyceride levels than their *Lep*$^{wt/wt}$ littermates. Though we could not elucidate the mechanism for these sex-specific differences, it is clear from our results that male *Lep*$^{ob/ob}$ mice are more severely impacted by lower circulating insulin levels than females. These findings are in line with the increased inflammation and reduced insulin sensitivity observed in adipose tissue of high fat-fed male as compared to female mice [25]. Furthermore, it has been previously demonstrated that female sex steroids protect against development of diabetes in mice [26,27]. Together, our results suggest that, in the absence of leptin, reduction of insulin levels has a greater impact on body weight, ectopic lipid accumulation, and body size of male as compared to female *Lep*$^{ob/ob}$ mice.

In leptin deficient mice, reduced insulin sensitivity is observed [28,29], and hyperinsulinemia precedes the onset of hyperglycemia [2,30,31]. We therefore examined the effects of reducing insulin alleles on fasting glucose, glucose tolerance, and insulin secretion. Reduction of insulin to circulating levels similar to *Lep*$^{wt/wt}$ levels resulted in rapid onset of hyperglycemia that was much more severe than that typically observed in *Lep*$^{ob/ob}$ mice [2,7,32]. These results suggest that, with reduced insulin gene dosage, hyperinsulinemia is curtailed, but leptin deficient

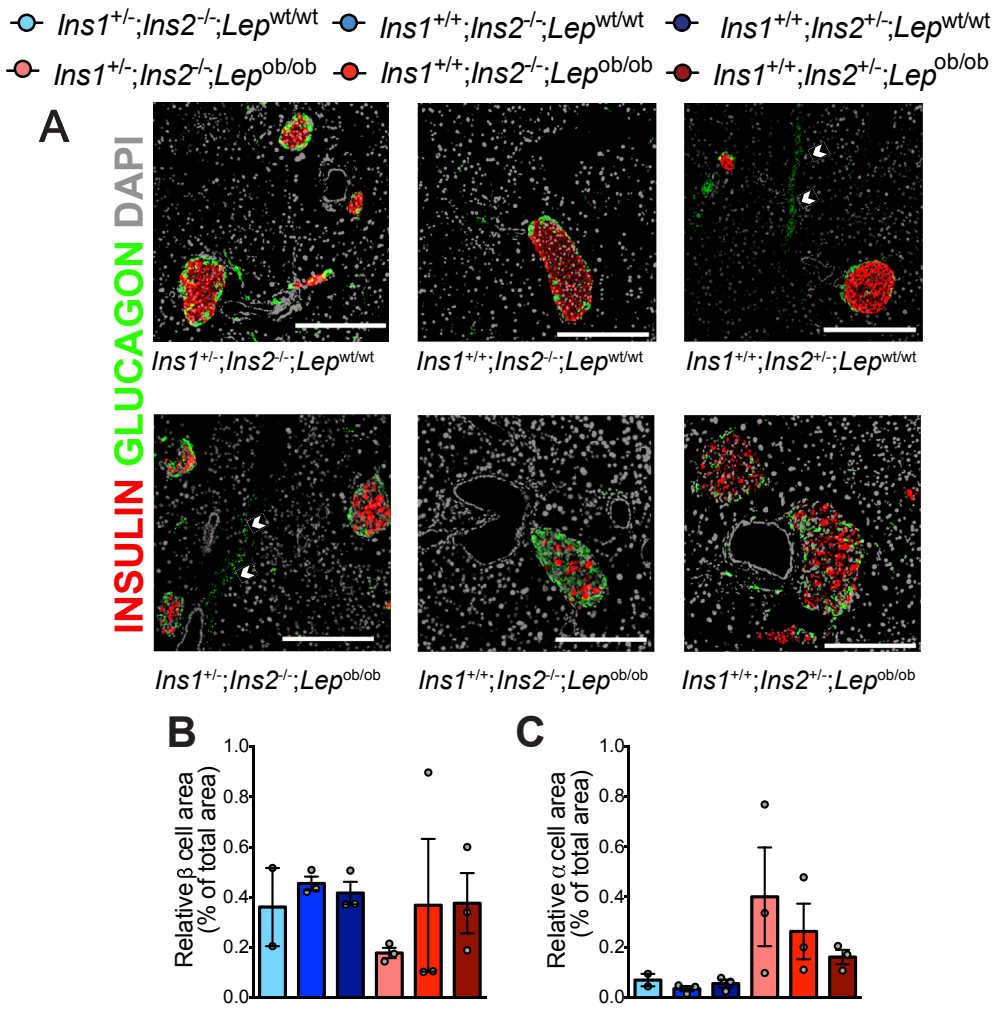

Figure 6: Representative pancreas sections (A) stained with insulin (red) and glucagon (green) from 8-week-old male mice, scale bar corresponds to 200 μm. White arrowheads indicate autofluoresence of red blood cells. Beta (B) and alpha (C) cell area relative to total pancreas area were quantified from pancreas sections.

mice are unable to compensate for increased insulin demand, resulting in hyperglycemia. Though euglycemia was maintained in *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice up to 7 weeks of age, male *Ins1*$^{+/+}$;*Ins2*$^{+/-}$;*Lep*$^{ob/ob}$ mice eventually developed hyperglycemia by 8 weeks of age. Thus hyperinsulinemia is able to prevent hyperglycemia transiently in this extreme model of obesity, but aging *Lep*$^{ob/ob}$ mice with reduced insulin gene dosage are unable to keep up with higher insulin demands, resulting in hyperglycemia. Despite loss of 2 or 3 insulin alleles, *Lep*$^{wt/wt}$ mice are euglycemic, suggesting that leptin plays a significant role in maintaining normal glucose levels. These results are consistent with studies in which leptin therapy is able to restore euglycemia in STZ-treated rodents [33–35] or rodent models of type 2 diabetes [36,37]. Given the glucose-lowering effects of leptin [38,39] and its role in regulation of insulin [5,40] and insulin sensitivity [41–43], the lack of leptin in *Lep*$^{ob/ob}$ mice may increase insulin demand as a compensatory mechanism to maintain euglycemia.

Despite reduced insulin gene dosage, glucose tolerance was normal, and relative alpha and beta cell area were similar across all *Lep*$^{wt/wt}$ groups. We observed a trend for decreased relative beta cell and increased alpha cell area in *Lep*$^{ob/ob}$ mice lacking 3 insulin alleles relative to *Lep*$^{wt/wt}$ mice lacking 1 insulin allele. Hyperglucagonemia has been implicated as a contributor to hyperglycemia in mice lacking leptin signaling [35,44]; thus, we hypothesized that it may contribute to the elevated glucose levels observed in *Lep*$^{ob/ob}$ mice. However, glucagon levels of *Lep*$^{ob/ob}$ mice were not significantly higher than *Lep*$^{wt/wt}$ mice and did not correlate with the degree of glycemia observed in *Lep*$^{ob/ob}$ mice, suggesting that elevated glucagon levels were not the major cause of hyperglycemia.

5. CONCLUSION

In summary, our findings highlight the important role of hyperinsulinemia in regulation of adiposity and maintenance of euglycemia in the absence of leptin action. Though incidence of complete leptin deficiency is rare in humans, the present study uses a model of extreme obesity to demonstrate the compensatory role required by insulin to achieve euglycemia in the absence of leptin. Our findings suggest that with reduced insulin gene dosage, *Lep*$^{ob/ob}$ mice have reduced ability to store excess glucose into fat and are unable to compensate for reduced insulin sensitivity. This results in decreases in fat mass and simultaneous development of hyperglycemia. Together, these findings suggest that in the absence of leptin, hyperinsulinemia promotes obesity and is also a necessary response used to achieve euglycemia in young *Lep*$^{ob/ob}$ mice.

DISCLOSURE STATEMENT

The authors have nothing to disclose.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.09.007.

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