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Prolactin activation of the long form of its cognate receptor causes increased visceral fat and obesity in male as shown in transgenic mice expressing only this receptor subtype

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Abstract

To date the best defined function of prolactin (PRL) is its action on the ovary and mammary gland, although it has also been shown to have an effect on lipid metabolism. Using mice engineered to express only the long form of the prolactin receptor (PRL-RL), we demonstrate that PRL acting through PRL-RL alone causes severe adipose accumulation in visceral fat of males at 6 months of age. The increase in visceral fat accumulation is attributed to loss of adipose-derived leptin, which results in diminished lipolysis. The reduction in leptin also corresponds to decreased activation of AMP-activated protein kinase (AMPK), which further results in diminished fatty acid oxidation and increased fatty acid synthesis. Interestingly, the blunted AMPK response was only observed in adipose tissue and not in liver suggesting that this PRL mediated effect is tissue specific. A glucose tolerance study inferred that PRL-RL mice may suffer from insulin resistance or a reduction in insulin production that is not due to aberrant expression of glucose transporter 4 (Glut4). Collectively, our findings demonstrate that PRL signaling through the long form receptor causes reduced fatty acid oxidation, increased lipid storage, glucose intolerance, and obesity. These findings are of great importance towards understanding the etiology of obesity associated with hyperprolactinemia in humans as well as the role of PRL as a metabolic regulator in adipose tissue.

Keywords

Prolactin; adipose; obesity; and AMP-activated protein kinase

Introduction

Obesity and metabolic syndrome are influenced by numerous variables such as genetic predisposition, diet, and exercise. It is well established that hormones such as leptin, growth hormone, and estrogen play a role in maintaining metabolism and aberrant hormone signaling can lead to obesity or insulin resistance [1–3]. Prolactin (PRL) is a pleiotropic hormone that is produced and secreted by lactotrophe cells in the anterior pituitary. Although it is well established that PRL plays a major role in female reproduction, little is known about the non-reproductive functions of PRL, in particular its role in metabolic tissues such as adipose. Recently, PRL was discovered to be secreted by human, but not mouse, adipocytes [4–6] and previous investigations have shown that PRL receptors (PRLR) are expressed in adipose tissues [4, 7]. PRL has been shown to upregulate the expression of

its receptors and to stimulate adipocyte differentiation, indicating a role for PRL in lipid metabolism [4, 8]. However, there is large inconsistency in the data addressing the function of PRL in adipose tissue. PRL has been shown to inhibit lipolysis in rat and human, but not mouse adipose tissue explants [7, 9]. Recently, PRL has also been found to suppress malonyl-coA expression suggesting that PRL inhibits lipogenesis [10]. Similarly, it is inconclusive as to whether PRL stimulates or suppresses leptin release, an adipokine that controls food intake and energy expenditure. Leptin is low or unchanged in PRL-R null mice [11–12], but is also elevated in both PRL-overexpressing and PRL null mice [9, 13]. In humans, PRL inhibits lipoprotein lipase activity, an enzyme that hydrolyzes lipids into fatty acids [14], and adiponectin, an adipokine involved in glucose regulation and fatty acid catabolism [15]. Given that PRL may play a role in lipid metabolism and that high levels of PRL positively correlate with obesity, PRL appears to be a metabolic modulator, acting via an unknown mechanism [16–19].

PRL signals through PRLR, a member of the cytokine class I family of receptors that lack intrinsic kinase activity. Alternative splicing of the single PRLR gene produces two types of receptors (short and long forms) in rodents. Expression of the PRLR isoforms varies from tissue to tissue. The adipose tissue expresses both the short (PRL-RS) and long forms of the receptor (PRL-RL), however, PRL-RL is expressed in much higher abundance [4, 7–8, 11]. PRL activates signal transducers and activators of transcription protein (STAT) 5 in adipocytes [8], an event that involves PRL-RL, but not PRL-RS [20–21]. This suggests that PRL-RL may be the functional receptor for PRL mediated action in white adipose tissue. The goal of the current investigation is to examine the role of PRL and that of its cognate receptor, PRL-RL, in adipocytes and in fat metabolism using transgenic mice expressing only PRL-RL.

Materials and Methods

Experimental Animals

Mice expressing only PRL-RL were previously generated by our laboratory to study the specific role of this receptor on ovarian function [22]. Briefly, transgenic mice were generated by overexpression of a transgenic construct carrying cDNA for PRL-RL driven by the EF1 promoter, which is active in all cells at every stage of differentiation. Mice were then backcrossed into the PRLR null mice [23] line to yield mice expressing only the PRL-RL isoform (PRL-RL). Mice were housed under standard conditions of controlled light (0700–1900 h) and temperature (22–24°C) with free access to standard rodent chow and water. All experimental procedures were performed in accordance to the Guidelines of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Only male mice were studied for the present investigation. Male mice were euthanized by cervical dislocation and body weight and visceral fat wet weights were recorded. Testis and epididymal fat pads were collected and processed for histological analysis as previously described [24–25].

RNA Isolation and semi-quantitative PCR

RNA was isolated from adipose tissue using TRIzol (Invitrogen) according to manufacturer's protocol. 1 µg of RNA was reverse transcribed to cDNA resulting in 20 µl of product which was then diluted to 100 µl final volume and 3 µl of diluted product was subsequently used for Q-PCR analysis using Fast Sybr Green Master Mix (Applied Biosciences). Primers for PRLR were adapted from [26]. L19 was used as an internal control for Q-PCR reactions. Primers for PPAR, SREBP1, adiponectin, IGF-1, leptin, and hormone sensitive lipase were adapted from [8].

Glucose Tolerance Test

Mice were fasted for 12 hours by removal of food and a fresh changing of bedding. Water was provided ad libitum during the fasting period. Fasted blood glucose levels were measured the prior to the experiment. Mice tails were slightly nicked with a razor blade and one drop of blood was collected directly on a blood glucose test strip and was measured by Breeze2 blood glucose monitoring system (Bayer). For the experiment, male mice were separated one per cage and injected with 1000mg/kg glucose (50% Dextrose solution). This dose has been successfully used in the past to determine glucose tolerance in mice [27–28]. Blood glucose was monitored every 15 min from a single drop of blood from the tail. Approximately 120 min post-injection, mice were re-housed and fed normal chow.

Western Blot

Adipose and liver lysates were prepared by homogenization of the tissue in RIPA buffer (Boston BioProducts) containing 1X protease inhibitor cocktail (Calbiochem), EDTA, and 1mM sodium orthovanadate. Protein concentration was measure by BCA assay (Thermo Fisher Scientific/Pierce Protein Research Products). 25µg of total protein was separated on a 10% SDS-PAGE gel and subsequently transferred to Immobilon PVDF membrane (Millipore). Western blotting was performed as previously described [20]. Blots were analyzed using a Supersignal West Pico chemiluminescence detection system (Thermo Fisher Scientific/Pierce Protein Research Products) and exposed to autoradiographic film (GE Healthcare). Antibodies against phosphorylated and total AMPK and AMPK were purchased from Cell Signaling Technology. Antibody against Glut4 and actin were purchased from Santa Cruz Biotechnologies and Abcam, respectively.

Statistical Analysis

Data were examined by t test, one-way ANOVA followed by the Tukey post test, and two-way ANOVA followed by Bonferroni post test using Prism software (GraphPad Software Inc.). Values are considered statistically significant at * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Results

Characterization of Male Mice Expressing only PRL-RL

As shown in Fig. 1A, we first established that PRL-RL expression is successfully restored in adipose tissue of PRL-RL transgenic mice. In contrast to the PRLR null mice where this receptor is not detectable in adipocytes, PRL-RL is expressed at normal levels in transgenic mice. We found the PRL-RL expressing males to be fertile and to have no apparent testicular defects as compared to both wild type and PRLR null (PRLR^{-/-}) mice (Fig. 1B). However, by 6 months of age, male PRL-RL mice are noticeably larger than wild type counterparts (Fig. 1C). Body weight measurement of wild type, PRLR^{-/-}, and PRL-RL revealed no difference in body weight at 3 and 6 months of age between the groups (Fig. 2A). Postmortem examination of the mice at 6 months revealed that PRL-RL mice had markedly more epididymal/visceral fat as compared to wild type and PRLR^{-/-} littermates (Fig. 2B). The difference in epididymal fat pad weight is apparent at 6, but not 3 months of age (Fig. 2C). The increase in lipid accumulation appears to be due to an enlargement in adipocyte cell size as determined by histological examination (Fig. 2D).

Ubiquitous Expression of PRL-RL Leads to Glucose Intolerance or Insulin Insensitivity

Insulin resistance is often associated with obesity. Therefore, we examined whether PRL-RL mice had a normal insulin response. PRL-RL mice had normal fasted glucose levels as compared to wild type and PRLR^{-/-} mice (Fig. 3A). However, when challenged with

glucose, PRL-RL mice showed impaired glucose tolerance (Fig. 3B). These results suggest that these transgenic mice are either insulin resistant or have defective insulin secretion. This impaired glucose response is not due to a defect in Glut4 expression, the major glucose transporter in the adipose tissue, compared to total protein expression (Fig. 3C).

qPCR Analysis of Factors Involved in Adipocyte Differentiation and Metabolism

We examined by real time qPCR (qPCR) for marked changes in several factors that may contribute to the increased fat deposition in mice with ubiquitous expression of PRL-RL (Fig. 3D). Expression of PPAR, a master regulator of adipocyte differentiation and that of ADD1/SREBP1, a transcriptional regulator of genes involved in lipid synthesis, were unchanged in PRL-RL expressing mice as compared to wild type. There was also no difference in expression of hormone sensitive lipase, IGF-1, or adiponectin (Fig. 3D); however, there was a significant decrease in leptin expression (Fig. 3D, bottom right panel). Low levels of leptin indicate that these mice have an increase in food intake and suppression in energy expenditure which could tip energy balance in favor of fat accumulation.

Changes in Adipocyte Metabolic Pathway

One enzyme that plays a critical role in regulating energy homeostasis is AMP-activated protein kinase (AMPK). Because leptin has been shown to activate the AMP-activated protein kinase (AMPK) pathway to stimulate fatty acid oxidation in skeletal muscle, AMPK activation was examined. AMPK, when phosphorylated and activated, inhibits acetyl CoA carboxylase (ACC) and prevents the formation of malonyl CoA necessary to drive fatty acid synthesis. Activation of AMPK requires phosphorylation of the Thr172 residue on AMPK, as well as Ser108 phosphorylation on AMPK subunit. Phosphorylation of AMPK (Fig. 4A) and AMPK (Fig. 4B) was significantly lower in adipose tissue of PRL-RL mice. This effect was specific to adipocytes since no change in AMPK activation was detected in liver (Fig. 4C). This suggests that ubiquitous expression of PRL-RL in epididymal fat causes a decrease in AMPK activation and ultimately leads to ACC driven fatty acid synthesis resulting in increased epididymal fat storage.

Discussion and Conclusions

In the present investigation, transgenic mice expressing only PRL-RL were used to study the role of PRL signaling through this receptor as a possible metabolic regulator in adipose tissue. Since hyperprolactinemia is linked with infertility and obesity in men (rev. in [29]) and [16–19], PRL-RL male transgenic mice were first examined for these defects. Although the primary cause of infertility in men with hyperprolactinemia is due to sexual dysfunction/impotence, other studies, in rats, have demonstrated that hyperprolactinemia-induced suppression of follicle stimulating hormone (FSH) can lead to poor sperm quality [30]. While it has been suggested that PRL plays a role in male fertility [31], deletion of PRL or PRL receptor had no impact on male fecundity in mice. Therefore, it was not surprising to discover that male PRL-RL mice have normal testes, displayed normal mating behaviors, and are fertile.

Although no phenotypic differences were observed between the male mice at an early age, by 6 months, PRL-RL male mice were noticeably larger in size as compared to wild type and PRLR^{-/-} mice. Our finding that visceral fat depots in PRL-RL male mice are critically larger is of great clinical importance given that obese men secrete higher levels of PRL from visceral fat than women [5]. Importantly, patients treated with antipsychotic drugs that suppress dopamine and elevate PRL, report obesity, hyperglycemia and dyslipidemia [32–33]. Treatment with bromocriptine, a dopamine agonist which blocks PRL release, leads to weight loss [34] and ameliorates dyslipidemia [35] in a genetic mouse model of obesity (ob/

ob mice) as well as in humans [36]. Taken together, these data indicate that PRL is a potent regulator of adiposity. Since PRL secretion from visceral fat is higher in obese men and since PRL can stimulate the expression of PRL-RL in adipocytes [4, 8], it is conceivable that one mechanism of PRL-mediated obesity is that PRL may stimulate PRL-RL expression leading to an increase in adiposity in visceral fat stores.

A major limitation in the field is that rodent models employed to study the role of PRL in adipose tissue function has resulted in conflicting data. One study determined that deletion of the PRLR gene leads to a decrease in fat deposition in knockout mice by 16 weeks of age [11]. To the contrary deletion of PRL led to no difference in body weight despite chow of low or high fat diet [9]. One possible explanation for the discrepancy in the literature may be due to the genetic background of the mice. PRL null mice were generated on the background of the C57BL6 mice, whereas the PRLR gene was deleted in the SV129 mouse background. Given the difference in mouse strain, the propensity to develop metabolic disorder and the severity of disease may manifest differently due to genetic variance [37–39]. Our findings clearly indicate that PRL acting through PRL-RL alone can stimulate adipocyte hypertrophy in mice. This is in agreement with the fact that PRL stimulates adipocyte differentiation in cultured mouse adipocytes [8]. The increase in epididymal fat in PRL-RL mice is correlated with an obesity driven glucose intolerance as seen by our glucose tolerance tests. Whether this is insulin resistance is due to a defect in insulin secretion or insulin responsiveness remains to be determined. One of the important regulators of insulin response in metabolic tissues such as adipose and skeletal muscle is Glut4. Glut4 is down regulated in adipose tissue, but not skeletal muscle, under obese and diabetic conditions in both humans and rodents [40–41]. We have demonstrated that Glut4 expression, the major glucose transporter in adipose, does not change in adipose tissue from PRL-RL mice indicating that insulin resistance is not due to a Glut4 expression defect in adipose tissue.

Given the enlargement in fat depot and adipocyte size in PRL-RL male mice, we examined whether there were any changes in known markers of adipocyte differentiation and lipid metabolism. Our finding that leptin, an adipokine known to suppress food intake and increase energy expenditure, transcript level was significantly decreased supports the obesity phenotype in these mice and is in agreement with a previous investigations [42]. We also examined the activation status of AMPK, a well established energy sensor within cells. When phosphorylated, AMPK directly inhibits acetyl CoA carboxylase and prevents the formation of malonyl CoA in turn leading to fatty acid oxidation and inhibition of lipogenesis (rev. in [43]). The finding that AMPK phosphorylation/activation is severely diminished at both the α and β subunits in adipose tissue, but not liver, strongly suggests that there is a tissue specific impairment in fatty acid oxidation and an increase in lipogenesis in PRL-RL expressing mice. Although it has been recently shown that PRL can activate fatty acid oxidation, via AMPK and carnitine palmitoyl transferase 1, in human breast cancer cell lines [44], these cell lines express both long, short, and intermediate isoforms of the receptor [45]. Currently, while it is becoming more accepted that the different isoforms of PRLRs can heterodimerize [46–47], it is unknown if heterodimerization can impact the signaling mechanism of PRL. The function of PRL-RS in white adipose tissue remains to be determined. Taken together these data strongly suggest that PRL acting through PRL-RL alone is an inducer of lipogenesis and fat accumulation by reducing AMPK activity.

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Figure 1. Transgenic mice expressing only PRL-RL are fertile, but are larger in size

A) Quantitative PCR analysis of PRLR expression in adipose tissue of wild type, PRLR-/-, and PRL-RL transgenic mice. Graph is representative of an n=3 mice per group. B)

Histological examination of testes structure of wild type, PRLR-/-, and PRL-RL mice with an n=3 mice per group. C) Image of wild type, PRLR-/-, and PRL-RL mice at 6 months of age.

Figure 2. PRL-RL male mice have an increase in epididymal fat

A) Body weight measurement of wild type, PRLR^{-/-}, and PRL-RL mice at 3 and 6 months of age with an n=4–8 mice per group. B) Representative image of epididymal fat pad of wild type, PRLR null, and PRL-RL mice at 6 months of age. # and † designate statistical significance reaching p<0.05 between wild type and PRL-RL and statistical significance reaching p<0.01 between PRLR null and PRL-RL mice, respectively. C) Weight of epididymal fat pads at 3 and 6 months of age from wild type, PRLR null, and PRL-RL with an n=4–8 mice per group. D) Hematoxylin and eosin staining of epididymal fat of PRLR null and PRL-RL mice at 6 months of age. Images are representative of an n=3 per group.

Figure 3. PRL-RL male mice are glucose intolerant

A) Fasted blood glucose levels. B) Glucose tolerance test of wild type, PRLR null, and PRL-RL mice at 6 months of age. C) Western blot analysis of Glut4 expression in adipose tissue of male wild type and PRL-RL mice. D) Real time PCR analysis for the expression of different markers in adipose tissue from wild type and PRL-RL mice. All data is representative of an n=4 mice per group.

Figure 4. Reduction in AMP-kinase activation in adipose, but not liver of PRL-RL mice
 Western blot analysis of AMPK (A) and AMPK (B) activation in adipose tissue of wild type and PRL-RL mice. C) Western blot of AMPK activation in liver tissue of wild type and PRL-RL mice. All data is representative of an n=4 mice per group.