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PI3K signaling in leptin receptor cells: role in growth and reproduction

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Running head: PI3K role in growth and reproduction

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Abstract

Nutrition and growth are important signals for pubertal development, but how they are perceived and integrated in brain circuits has not been well defined. Growth hormones and metabolic cues both recruit the phosphatidylinositol 3-kinase (PI3K) signaling in hypothalamic sites, but whether they converge into the same neuronal population(s) is also not known. In this review, we will discuss recent findings from our laboratory showing the role of PI3K subunits in cells directly responsive to the adipocyte-derived hormone leptin in the coordination of growth, pubertal development and fertility. Mice with deletion of PI3K p110α and p110β catalytic subunits in leptin receptor cells ($LR^{\Delta\alpha+\beta}$) have a lean phenotype associated with increased energy expenditure, locomotor activity, and thermogenesis. The $LR^{\Delta\alpha+\beta}$ mice also show deficient growth and delayed puberty. Deletion of a single subunit (i.e., p110α) in LR cells ($LR^{\Delta\alpha}$) cause a similar phenotype of increased energy expenditure, deficient growth, and delayed pubertal development indicating that these functions are preferably controlled by p110α. The $LR^{\Delta\alpha}$ mice show enhanced leptin sensitivity in metabolic regulation but, remarkably, these mice are unresponsive to leptin's effects on growth and puberty. The PI3K is also recruited by insulin, and a subpopulation of LR neurons are responsive to intracerebroventricular insulin administration. Deletion of insulin receptor (InsR) in LR cells causes no changes in body weight or linear growth, and induces only mild delay in pubertal completion. Our findings demonstrate that PI3K in LR cells plays an essential role in growth and reproduction. We will also discuss potential neural pathways underlying these effects.

Key words: hypothalamus, metabolism, reproduction, insulin, bone

Introduction

The role of metabolic cues in pubertal progression has been well described in different mammalian species (1-4). Among these cues, the adipocyte-derived hormone leptin plays a critical role. The circulating levels of leptin are positively correlated with the amount of body fat (5-7). Disruption of leptin signaling causes obesity, diabetes and a number of neuroendocrine dysfunctions including changes in bone accrual and growth, pubertal arrest and infertility (5, 8-16, 17 Farooqi, 2007 #910). Genetic mutations of leptin or leptin receptor in humans are rare (11, 18, 19). Obese individuals exhibit hyperleptinemia with low or absent response to leptin administration (20, 21). The underlying mechanisms are diverse, but impairment of leptin receptor signaling seems to play a major role. Therefore, leptin "resistance" due to disruption of leptin receptor (LR) function or signaling capacity may cause most of the metabolic and neuroendocrine deficits observed in obese subjects (22, 23). Excess adiposity and high leptin levels also impact the reproductive function and growth by disrupting steroidogenesis in males and females, aggravating ovulatory disorders, and altering bone formation (17, 23-27). The increasing prevalence of childhood obesity has been associated with early puberty in girls (28-30). Thus, it has become clear that metabolic cues are key for the coordinated timing of growth and puberty and maintenance of a healthy reproductive function in adult life.

The LR is a member of the class I cytokine receptor family comprised of several isoforms (10, 31-35). The LR long form (LRb) contains three intracellular tyrosine residues that are phosphorylated by JAK2. Selective blockade of each one of these residues has produced compelling findings on the effects of specific signaling pathways in leptin function (36-38). For example, disruption of Tyr1138 or leptin-induced STAT3 signaling generates severely obese mice, with disruption of thyroid and adrenal axes. However, they show improved glucose homeostasis, growth and fertility compared to loss-of-function mutation of leptin or leptin receptor genes (i.e., *ob/ob* or Lep^{ob} and *db/db* or $Lepr^{db}$ mice). Lack of either leptin-induced SHP2-ERK (Tyr985) or STAT5 (Tyr1077) caused only mild metabolic or reproductive phenotypes (37-39).

Leptin also recruits phosphatidylinositol 3-kinase (PI3K) signaling (40-43), a major molecular pathway associated with metabolic regulation, insulin signaling, glucose homeostasis and neuroendocrine function (40, 42, 44-49). However, the requirement of PI3K in leptin responsive cells for growth and pubertal maturation had not been described until very recently (50). In this review, we will discuss findings showing that PI3K signaling functions as a key molecular pathway linking leptin and the neuroendocrine axes. Because PI3K is also recruited by insulin,

we will discuss its potential role in the integration of both metabolic cues (43, 51, 52).

PI3K in leptin action: Molecular components

Studies from different groups support the hypothesis that leptin recruits the PI3K signaling pathway to exert some of its effects (40, 42). For example, leptin hyperpolarizes a subset of arcuate nucleus neurons via activation of an ATP sensitive potassium channel, resulting in reduced firing rates. Inhibitors of PI3K blocked this effect (44, 53, 54). Similarly, leptin-mediated depolarizing effects on proopiomelanocortin (POMC) neurons are dependent on PI3K signaling (55). Pharmacological inhibition of PI3K blocked the ability of intracerebroventricular leptin to reduce food intake (40, 44), and precluded the effects of hypothalamic leptin administration to inhibit white adipose tissue lipogenesis (56).

Multiple classes of PI3Ks exist. In particular, class IA is primarily associated with metabolic regulation (57-59). PI3K class IA consists of heterodimers of one regulatory and one catalytic subunit. The regulatory subunits are typically five, often called the p85s, and the catalytic subunits comprise three variants referred to as p110s (57, 59). Activation of the heterodimers occurs when the p85 binds to the insulin receptor substrate (IRS) and position the catalytic subunit in the cellular membrane, where PI3K catalyzes the phosphorylation of the phosphatidylinositol bisphosphate (PIP2) to PIP3 that, in turn, recruits and activates downstream pathways.

The p110α and p110β subunits are ubiquitously expressed, whereas the p110γ is virtually absent in the brain (60, 61). Because of the distribution pattern and the lack of p110γ in hypothalamic sites, most of the studies in metabolic control have focused on the roles of p110α and p110β subunits. Mice lacking p110α or p110β die early during embryonic development (62, 63). However, mice carrying a *knockin* mutation causing a 50% loss-of-function of p110α activity (kinase-dead/D993A) are viable, but display suppressed IRS signaling, decreased responsiveness to insulin and leptin, reduced linear growth, hyperphagia and increased adiposity (47). Deletion of p110α in cells expressing POMC or steroidogenesis factor 1 (SF1) decreased energy expenditure and increased susceptibility to high-fat diet (64, 65).

These initial findings suggested that the p110α was the main isoform downstream of leptin or insulin action. However, subsequent studies showed that the isoforms interactions are more complex than previously anticipated. Insulin-induced phosphorylation of Akt (pAkt) is only partially blocked by inhibitors of either p110α or p110β, whereas the administration of both

inhibitors completely prevented insulin signaling. Likewise, the combined administration of p110α and p110β inhibitors blocked the acute anorexigenic action of leptin and insulin (66). Intact p110β or both p110α and p110β subunits in neurons expressing agouti-related protein (AgRP) seem to be required for metabolic control (67, 68). In SF1 neurons, distinct catalytic subunits are necessary for leptin or insulin cellular effects (69). Thus, it soon became clear that blockade of PI3K downstream of leptin would only be attained by deletion of both subunits. This concept is further strengthened by data showing that LR neurons coexpress both p110α and p110β subunits (70).

Effects of deletion of p110α and p110β subunits in LR neurons

To assess the direct role of PI3K downstream of leptin, we generated mice with deletion of both p110α and p110β catalytic subunits in LR cells (50, 70, 71). The experimental group was comprised of mice homozygous for three alleles: LR-Cre, p110α-floxed and p110β-floxed (48, 72, 73), named $LR^{\Delta\alpha+\beta}$. We used LR-Cre homozygous mice because in preliminary studies we found only partial deletion of PI3K in LR cells of heterozygous $LR^{Cre/+}$ mice harvested by FACS (fluorescence activated cell sorting) (50). This finding is also in agreement with previous studies using a different LR-Cre mouse line (74-76). To assess if Cre in homozygosity alters LR function, we performed a systematic evaluation of $LR^{cre/cre}$ mouse phenotype. No ectopic expression of Cre (using reporter genes), and no metabolic, growth or reproductive deficits were observed (50).

Male and female mice were evaluated separately due to the sexually dimorphic responses in metabolic and neuroendocrine functions (77). Precautions were taken to avoid inconsistencies and exogenous interference in the physiological measurements. For instance, on postnatal day 1 (P1), litter size was kept consistent (equal in number) among groups and cohorts to avoid early-life nutritional effects on body weight and metabolic programming (1, 4, 78). After weaning (P21), males and females were fed phytoestrogen-reduced diet to prevent the effects of exogenous estrogens on pubertal development and fertility. Body weights were measured weekly and the metabolic phenotype was monitored by CLAMS (Comprehensive Laboratory Animal Monitoring System) using average values obtained from 3-4 days of metabolic recordings to avoid individual variations or random errors.

Briefly, male and female $LR^{\Delta\alpha+\beta}$ mice showed decreased body weight, starting at 4-5 weeks of age, associated with decreased lean and fat mass and decreased linear growth. They also

showed increased food intake (when normalized by body weight), energy expenditure, locomotor activity and brown adipose tissue *Ucp1* expression, suggesting increased thermogenesis. Females, but not males, had delayed puberty determined by late vaginal opening (a sign of puberty onset) and delayed first estrus (puberty completion) (50). Whether disruption of estradiol actions due to PI3K imbalance in LR cells is the cause of the sexually dimorphic phenotype in reproductive function needs further evaluation.

The findings raised several questions that require additional studies. We initially focus on answering the following: a) Are individual PI3K catalytic subunits associated with specific physiological control? b) Is the delayed puberty in females caused by the decrease in body weight, fat mass or leptin levels?

Effects of deletion of p110α subunits in LR neurons

To address the initial question, we generated a mouse line with deletion of a single PI3K subunit, p110α, in LR cells. We chose p110α because of data from D993A mice showing that half dosage of p110α induces changes in metabolic and growth phenotypes (47). To avoid potential confounders originated from using different genetic backgrounds, the mouse colony with deletion of p110α in LR cells ($LR^{\Delta\alpha}$) was derived from the $LR^{\Delta\alpha+\beta}$ mice.

The $LR^{\Delta\alpha}$ male and female mice showed decreased body weight, lean and fat mass, and reduced linear growth. Changes in growth was observed only in adults (around and after P60); no differences were observed before or during pubertal maturation (P40). Low bone mineral density was detected in the femoral trabecular and cortical layers, associated with a decrease in hepatic growth hormone receptor (*Ghr*) and insulin-like growth factor 1 (*Igf1*) expression in adult females. Similar to $LR^{\Delta\alpha+\beta}$ mice, food intake was increased when normalized by body weight. Increased energy expenditure and delayed pubertal maturation were observed. Female $LR^{\Delta\alpha}$ mice also showed prolonged estrous cycles and progressive subfertility. No changes in locomotor activity and brown adipose tissue uncoupling protein 1 (*Ucp1*) gene expression was detected. Together, these findings indicate that apart from energy balance, the p110α subunit in LR cells is also necessary for typical growth and reproduction.

Because the $LR^{\Delta\alpha}$ (and the $LR^{\Delta\alpha+\beta}$) mice showed low body weight, fat mass and leptin levels, we performed several metabolic manipulations to assess if the delayed puberty and reproductive phenotypes were secondary to the metabolic disruption. We started by applying the early postnatal overnutrition approach via manipulation of the litter size (1, 4, 78). A cohort of mice

was maintained in small litters in the attempt to increase or normalize the body weight of $LR^{\Delta\alpha}$ mice prior to puberty onset. This manipulation was successful until weaning day, when $LR^{\Delta\alpha}$ mice start to consistently decrease the weight gain. Timing of pubertal development and leptin levels were marginally improved, not corrected. Again, because we still found a lean phenotype in the postnatal overnutrition paradigm after weaning, our question had not been solved. We decided to use the leptin challenge instead.

Several groups have shown that leptin treatment in small doses that do not alter metabolic responses advances puberty in rodents (79, 80). We used a very similar approach and found that, whereas control mice had early puberty onset, $LR^{\Delta\alpha}$ female mice were unresponsive to the effects of leptin on the timing of pubertal development. Another interesting finding yielded by this experiment was the increased leptin sensitivity in metabolic regulation of the mutant mice potentially due to the increased basal levels of pAkt, leptin-induced pSTAT-3 and decreased levels of the PIP3 phosphatase PTEN (50).

Of further interest was the increased expression of AgRP mRNA and peptide in fed female $LR^{\Delta\alpha}$ mice. The AgRP neurons are located in the arcuate nucleus and coexpress LR (45, 81-83). Ablation of AgRP neurons ($AgRP^{DTR}$ mice) ameliorates the metabolic and reproductive phenotypes of leptin-deficient *ob/ob* mice. Improved fertility was also observed in LR-deficient *db/db* mice with global deletion of *Agrp* gene, and deletion of LR in AgRP neurons alters fertility (84-88). Thus, changes in AgRP levels and/or signaling in $LR^{\Delta\alpha}$ mice may explain the increased food intake, and the disruption of growth and pubertal development observed in $LR^{\Delta\alpha}$ mice (87, 89). It is also in agreement with findings showing that PI3K is required for leptin actions on *Agrp* gene expression (45). Alternatively, ablation of PI3K may have affected the acute actions of leptin in the ventral premammillary nucleus (PMV), a hypothalamic site associated with leptin action in female reproductive function (70, 74, 90, 91).

Pros **and** ***cons*** **of using LR-Cre line as a metabolically relevant target**

The LR expression is observed in many peripheral tissues and in the brain, with high density in hypothalamic sites (10, 92, 93). Thus, whether the effects observed using the LR-Cre mice were due to deletion of PI3K in neurons or in other peripheral organs may seem unclear. In this regard, it is important to emphasize that studies using conditional deletion or re-expression of LR have shown that leptin's effects in the neuroendocrine axes are mediated by the brain (94-96). The deletion of LR from gonadotropes caused no changes in body weight, timing of

pubertal maturation or estrous cycle duration (97). We have also shown that LR expression only in gonadotropes is not sufficient to improve the metabolic or the reproductive phenotypes of the LR null mice (98). Similarly, initial studies have suggested that the reproductive deficits caused by the lack of leptin signaling are not mediated by the gonads (99), indicating the brain is the main target of leptin in neuroendocrine regulation. However, the $LR^{\Delta\alpha}$ mice phenotype may not be entirely related to disruption of leptin signaling. We could hypothesize that the lack of PI3K downstream of other hormones and/or growth factors in ovaries, for instance, have contributed to the $LR^{\Delta\alpha}$ mice phenotype. LR-Cre reporter gene is expressed in theca cells (98) and studies have suggested that insulin signaling in theca cells is associated with obesity-induced increase in estrous cycle length (100). Deletion of insulin receptor in theca cells or gonadotropes blocked this response allowing females to maintain normal estrous cycles in obese conditions (100, 101). Together, these findings suggest that the reproductive phenotype of the $LR^{\Delta\alpha}$ mice is not associated with deletion of PI3K in gonadotropes or theca cells. As discussed in previous sections, blunted PI3K signaling in LR cells of the PMV or the arcuate nucleus (i.e., those coexpressing AgRP) are prime candidates.

Leptin receptor is also expressed in the adipocytes, liver and osteoblasts (10, 102, 103). Whether deletion of PI3K subunits in LR cells of peripheral tissues may have impacted the growth and the reproductive phenotypes observed in our studies needs further evaluation. However, it should be noted that deletion of p110α only in adipocytes caused a very distinct phenotype from that observed in our studies, i.e., delayed puberty and infertility were observed only in male, not female, mice (104). The mechanism(s) associated with this phenotype is unknown. Moreover, no reproductive or linear growth deficits have been described in mice with deletion of LR or disruption of PI3K class I in the liver (105, 106).

Complexity of PI3K signaling and potential confounders

The PI3K signaling pathway is comprised of intricate interactions of independent subunits and molecular targets. Thus, genetic modifications of specific subunits may cause an imbalance of the entire complex, generating unexpected phenotypes. For examples, mice with deletion of p85α or p85β regulatory subunits have improved insulin sensitivity and hypoglycemia, despite the fact that PI3K is a key pathway for insulin effects in glucose homeostasis (58, 107, 108). The selective deletion of the p110 catalytic subunits in LR cells may have caused a similar response, i.e., improvement of leptin sensitivity in energy homeostasis due to increases in energy expenditure and in basal pAkt, decrease in body weight and the potentiation of leptin induced pSTAT3. Notably, whereas leptin's effects in metabolism were amplified in $LR^{\Delta\alpha}$ mice,

linear growth and reproductive function were compromised. These findings suggest that PI3K is a crucial downstream signal of metabolic cues to growth and reproductive neuroendocrine axes.

Another potential confounder is the expression of LR in neurons with distinct or opposite functions as, for example, the AgRP and POMC neurons in the arcuate nucleus. Previous studies have assessed the role of PI3K subunits in AgRP, POMC and other neuronal populations (i.e., SF1) (64, 65, 67, 69, 109). Because only subsets of these neurons express LR, the results are ambiguous. It is not possible to determine if the observed effects are associated with lack of leptin, insulin or growth factors (69, 110). By using the LR-Cre mouse model, we expect the data to be more specific generating insights into the direct effects of leptin-induced PI3K in physiology. Further studies will be necessary to dissociate the role of specific LR neurons in each phenotypic changes observed.

LR cells likely coexpress a number of receptors that also recruit PI3K signaling (e.g., receptor tyrosine kinases or GPCRs). Therefore, it is possible that the deletion of PI3K subunits has altered the signaling of other hormones and/or growth factors producing a phenotype unrelated to leptin action. As mentioned before, one key hormone is insulin. By deleting PI3K from LR cells, we may have blocked the actions of insulin in subsets of LR neurons. To test this hypothesis, we used the Cre-loxP system to delete InsR from LR cells.

Effects of deletion of InsR in LR cells

Previous studies using electrophysiological recordings have suggested that leptin or insulin target distinct POMC and SF1 neuronal populations (69, 110). However, lack of changes in membrane potential does not preclude other cellular responses, such as gene expression and/or posttranslational modifications. To assess if downstream targets of insulin-induced PI3K with genomic actions may be detected in LR neurons, we performed a colocalization study in LR reporter mouse. Mice were treated with intracerebroventricular insulin to avoid potential confounders of using peripheral insulin administration, and FoxO1 translocation or pAkt were identified in LR-Cre reporter neurons (50). Partial colocalization was detected, reinforcing the hypothesis that the effects of PI3K deletion in LR cells was a result of partial blockade of insulin signaling. To test this, we generated mice with deletion of InsR in LR cells, using the same LR-Cre mouse line bred with previously validated InsR-floxed mice (111). No deficits in growth and only minor changes in reproductive physiology was observed in the mutant mice. Females showed a mild delay in puberty completion, suggesting that lack of insulin signaling in LR cells

($LR^{\Delta InsR}$) may add to the effect observed in $LR^{\Delta\alpha}$ and $LR^{\Delta\alpha+\beta}$ mice that showed a more severe disruption of pubertal timing. Interestingly, whereas female $LR^{\Delta InsR}$ mice showed virtually no metabolic deficits, males had increased fat mass and glucose oxidation, but no reproductive deficits (50, 112). We concluded that the changes in metabolic, growth and reproductive phenotypes of the $LR^{\Delta\alpha}$ and $LR^{\Delta\alpha+\beta}$ mice are not due to blockade of insulin signaling. Because the conditional deletion of InsR in the brain causes metabolic and reproductive deficits (111), our findings also indicate that insulin actions in these physiological systems are attained by targeting cells distinct from those expressing LR. Whether other PI3K recruiting factors account for the effects observed in $LR^{\Delta\alpha}$ and $LR^{\Delta\alpha+\beta}$ mice need further evaluation.

Genomic screening in humans has identified the PI3K as a key signaling pathway associated with pubertal development (113). In agreement, our findings in mice show that PI3K signaling selectively in LR cells plays a major role. The decreased growth of $LR^{\Delta\alpha}$ mice and the higher sensitivity to leptin in metabolic regulation makes the PI3K a potential target in conditions of delay in growth and puberty in humans, and in hypothalamic amenorrhea associated with sustained negative energy balance.

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