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Phosphatidyl Inositol 3-Kinase Signaling in Hypothalamic Proopiomelanocortin Neurons Contributes to the Regulation of Glucose Homeostasis

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Abstract

Recent studies demonstrated a role for hypothalamic insulin and leptin action in the regulation of glucose homeostasis. This regulation involves proopiomelanocortin (POMC) neurons because suppression of phosphatidyl inositol 3-kinase (PI3K) signaling in these neurons blunts the acute effects of insulin and leptin on POMC neuronal activity. In the current study, we investigated whether disruption of PI3K signaling in POMC neurons alters normal glucose homeostasis using mouse models designed to both increase and decrease PI3K-mediated signaling in these neurons. We found that deleting p85 alone induced resistance to diet-induced obesity. In contrast, deletion of the p110 catalytic subunit of PI3K led to increased weight gain and adipose tissue along with reduced energy expenditure. Independent of these effects, increased PI3K activity in POMC neurons improved insulin sensitivity, whereas decreased PI3K signaling resulted in impaired glucose regulation. These studies show that activity of the PI3K pathway in POMC neurons is involved in not only normal energy regulation but also glucose homeostasis.

Diabetes rates along with obesity are rising in the United States (1,2

Both insulin and leptin activate the phosphatidyl inositol 3-kinase (PI3K) intracellular signaling pathway ([18](#)). PI3K consists of an 85-kDa regulatory (p85) and a 110-kDa catalytic (p110) subunit ([19](#)), each having several isoforms. p85 binds insulin receptor substrate (IRS) molecules and localizes catalytic activity to the cell membrane, In which p110 phosphorylates phospatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), activating downstream molecules that bind PIP3 such as Akt. Reduced gene dosage of the catalytic subunit, as occurs in p110 inactivated cells or tissues, causes a severe reduction in insulin-stimulated PI3K activity, pAkt, and the phosphorylation levels of downstream components such as Forkhead box O-transcription factors (20,21). However, genetic inactivation of the regulatory subunit paradoxically increases signaling downstream of PI3K (22). This increase seems to result from both a compensatory increase in p85 expression (23) and reduced phosphatase and tensin homolog deleted from chromosome 10 (PTEN) activity because p85 forms part of the PTEN-activating complex (24). PTEN dephosphorylates PIP3, antagonizing the action of PI3K. Therefore, the loss of p85-induced Akt activation is due, in part, to decreased PTEN activity protecting the PIP3 pool produced by the remaining p110-p85 heterodimers.

Hypothalamic proopiomelanocortin (POMC) neurons are essential for normal body weight homeostasis (25,26,27,28,29) and may be important for glucose homeostasis as well. Centrally administered melanocortin agonists inhibit insulin release and alter glucose uptake and production (10). Furthermore, POMC-specific deletion of suppressor of cytokine signaling (SOCS)-3, a negative regulator of both leptin and insulin signaling, results in improved glucose homeostasis and insulin sensitivity (30). We recently showed that deleting both p85 and p85 from POMC neurons eliminates insulin and leptin effects on POMC neuronal activity (31). We therefore investigated whether normal glucose homeostasis requires POMC PI3K signaling using mouse models designed to either increase or decrease PI3K activity in these neurons. Using the cre/loxP system, we investigated mice lacking p85 in POMC neurons (up-regulation of PI3K mediated signaling) and mice lacking p110 in POMC neurons (down-regulation of PI3K mediated signaling).

Materials and Methods

Animal care

Care of all animals and procedures was approved by the University of Texas Southwestern Medical Center (*pik3CA POMCKO* studies) and the Beth Israel Deaconess Medical Center (*pik3r1 POMCKO* studies) Institutional Animal Care and Use Committees. Mice were housed in a temperature-controlled environment in groups of two to four at 22–24 C using a 14-h light, 10-h dark cycle. The mice were fed either standard chow (4% fat mouse/rat diet 7001; Harlan-Teklad, Madison, WI) or high-fat diet (88137 Western diet; Harlan Teklad), and water was provided *ad libitum* unless noted otherwise. Mice were killed by CO_2 narcosis.

Generation of mouse lines

Pomc-Cre mice [FVB background (26)] were mated with *pik3r1*$^{loxP/loxP}$ mice [129/Sv-C57BL/6-FVB mixed background (32)] or *pik3CA* $^{loxP/loxP}$ mice [129/SvJ-C57BL/6 mixed background (21)]. Breeding colonies were maintained by mating *pik3r1*$^{loxP/loxP}$ mice with *pik3r1 POMCKO* mice and *pik3CA* $^{loxP/loxP}$ with *pik3CA POMCKO* mice. Thus, littermate controls with the same genetic background as experimental animals except for POMC-cre expression were used for all experiments. Any mouse that tested positive for deletion of the *pik3r1* or *pik3CA* gene in tail tissue was excluded from all studies. Genotyping was performed according to protocols described previously (21,31).

Immunohistochemistry and *in situ* hybridization

Fed male 10-wk-old mice were perfused with 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO), and frozen coronal sections were cut at 25 Ëm (1:5 series) on a microtome. Sections were processed as reported previously from our laboratory (33).

To create the p85. and p110. cRNA probes (34), we used RT-PCR amplification. The first-strand cDNA was obtained using total rat brain RNA (Ambion, Inc., Austin, TX) as a template. For the p85 . probe, the cDNA was then amplified by PCR using the 5;primer: 5 ;-AGA ACG GCT ATC GAA GCA-3;corresponding to nt 1997-2015 and the 3;primer: 5 ;-GAC GCA ATG CTT GAC TTC-3;corresponding to nt 2552-2569 of p85 .. The region bounded by these primers is 572 bases and corresponds to the COOH-terminal Src homology 2 domain of p85 .. For the p110. probe, the cDNA was then amplified by PCR using the 5; primer: 5 ;-TGC GCT GGG TAC TGC GTG GC-3;corresponding to nt 2701-2720 and the 3 ;primer: 5 ;-TAC GTT CAA AGC ATG CT-3;corresponding to nt 3191-3207 of p110.. The region bounded by these primers is 506 bases. The amplification products were gel purified and cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA), by using standard techniques according to the manufacturer's protocol. The inserts of positive clones were verified by DNA sequencing with primers specific to the vector, T7 Sequenase (Amersham Life Science, Inc., Arlington Heights, IL), and the Sequetide system from NEN Life Science Products (Boston,

Evaluation of glucose homeostasis

Glucose in tail blood was assayed using a glucometer (One-Touch Basic; Lifescan, Milpitas, CA), with postprandial measurements taken at approximately 1000 h. Where indicated, blood was obtained from mice fasted for 16 h. For insulin tolerance tests, 3 h-fasted animals were injected ip with 1 mU/g human insulin (Humulin R; Eli Lilly Corp., Indianapolis, IN), and blood glucose values were measured immediately before and 15, 30, 45, 60, 90, and 120 min after injection. For glucose tolerance tests, overnight-fasted mice were injected with D-glucose (1 mg/g body weight), and blood glucose was measured immediately before and 15, 30, 45, 60, and

antibodies were a 1:1,000 dilution of the antibody against phospho-Akt (serine-473; Cell Signaling Technology, Beverly, MA), and a 1:10,000 dilution of the antibody against -actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistics

The data are reported as mean ± SEM. Comparisons between two groups were made by unpaired two-tailed *t* tests. Comparisons over time were carried out by calculating area under the curve for blood glucose levels or by repeated-measure ANOVA for body weight curves. $P < 0.05$ was considered to be

As previously mentioned, POMC neurons have been shown to regulate glucose homeostasis (10,30). We therefore went on to examine the effects of altered PI3K activity in POMC neurons on glucose

respectively, in POMC neurons. We found that although both groups maintained glucose tolerance, *pik3r1 POMCKO* displayed an increase in insulin sensitivity, and *pikCA POMCKO* mice displayed a decrease in insulin sensitivity as determined by basal insulin levels, insulin tolerance testing, and/or hyperinsulinemic euglycemic clamping.

Previous studies using targeted perturbation in POMC neurons largely supported a role for POMC PI3K signaling in glucose homeostasis, although definitive evidence has been lacking. Mice lacking 3-phosphoinositide-dependent protein kinase-1, an enzyme downstream of PI3K, in POMC neurons initially displayed impaired glucose metabolism (46). However, these results were confounded by severe hypocortisolism caused by loss of POMC-expressing corticotrophs in the pituitary. In addition, selective deletion of SOCS-3, a negative regulator of leptin and insulin signaling, in POMC neurons led to a significant improvement in glucose homeostasis and insulin sensitivity without any apparent impact on the adrenal axis (30). However, mice lacking insulin receptors in POMC neurons did not show any defect in glucose or energy homeostasis (47). Mice with POMC-specific disruption of PTEN, expected to result in constitutively high levels of PI3K signaling, were apparently not evaluated for insulin levels or glucose regulation (48). We previously examined the effect of simultaneous deletion of both regulatory subunits, p85   and p85 , from POMC neurons by crossing *pik3r1 POMCKO* mice with mice germline knockout for pik3r2 (31). We found that concurrent loss of these two p85 subunits in POMC neurons abolishes the ability of these neurons to respond to leptin or insulin stimulation, but any effect on glucose homeostasis could not be evaluated due to increased insulin sensitivity in the global p85 -null mice. Our current data establish that alteration of PI3K activity in POMC neurons can modulate hepatic insulin sensitivity and glucose regulation, providing a mechanistic link between the response of POMC neurons to leptin or insulin and their effects on glucose homeostasis (30,45).

In the current experiments, we also observed an effect of POMC neuronal PI3K activity on body weight regulation. This finding extends our prior study of the effect of PI3K regulatory subunits on body weight (30). Whereas ablation of both p85   and p85   subunits in POMC neurons was required to impair POMC neuronal activity in response to insulin and leptin as well as acute leptin-induced feeding, no obvious impact on long-term body weight regulation was observed in those mice (31). The contrast to the current results may arise from the fact that global deletion of p85   has been reported to reduce mouse size and affect insulin sensitivity (49). Thus, it is possible that the experimental animals were protected from developing obesity in the initial study. This interpretation is in agreement with results from mice lacking SOCS-3 in POMC neurons showing a reduction in weight gain on a high-fat diet (30). In addition, Plum *et al.* (48) have shown that POMC-specific disruption of PTEN results in hyperphagia, male obesity on normal chow, and female obesity on high-fat chow. However, interpretation of the latter results is complicated by morphological changes in POMC neurons and the existence of other downstream targets of PTEN, including MAPK and Shc (50,51,52,53,54).

Our results show that deletion of a single p85 isoform results in resistance to diet-induced obesity in female mice, possibly because the well-known anorexigenic effects of estrogens may be enhanced through increased PI3K signaling in POMC neurons (55,56,57). In addition, mice with increased PI3K activity in POMC neurons diverged from control values over time, whereas the body weight of mice with reduced PI3K signaling in these neurons tended to converge with control values. This observ(es. T 8 TD1.n).6(d)edd.6(n)0y on

In contrast, p110   does not appear to play a significant ro

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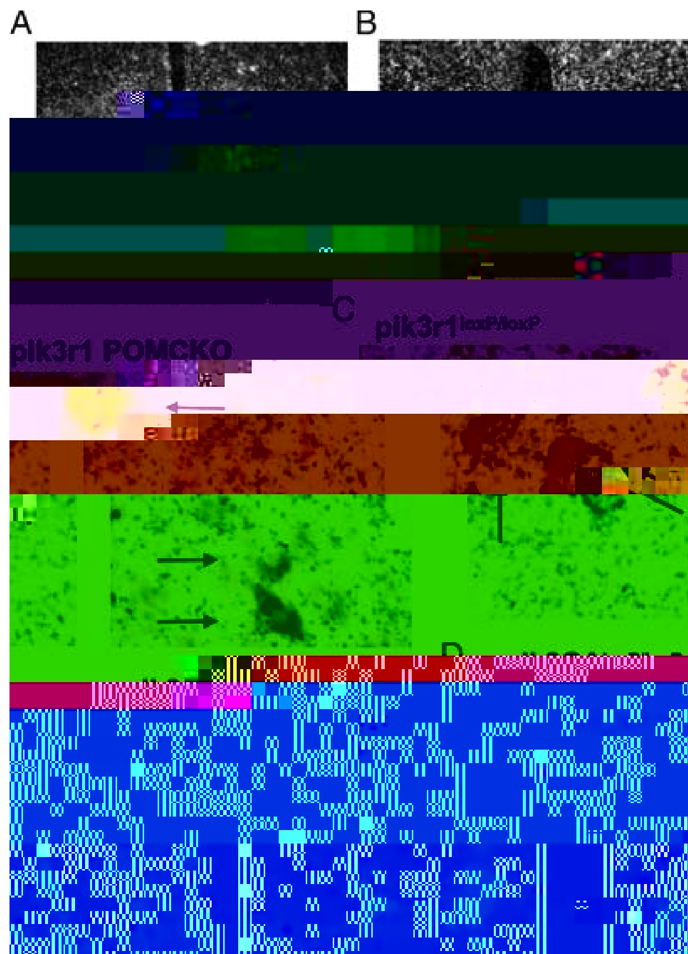
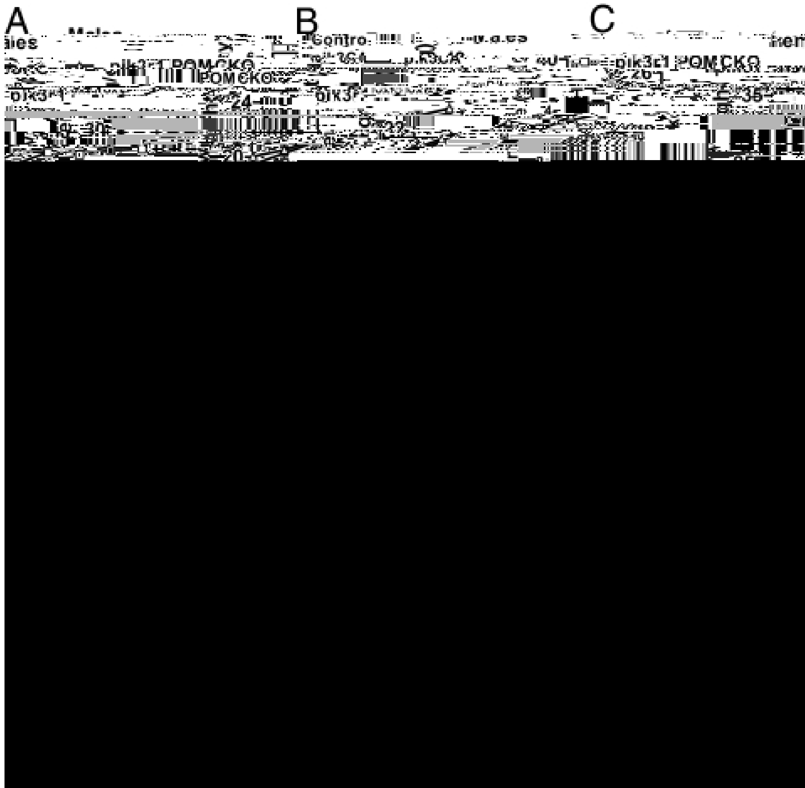
Figure 1



Targeted p85 or p110 deletion in POMC neurons. Photomicrographs of hypothalami from 3-month-old wild-type mice hybridized with probes for p85 (A) or p110 (B). Mice expressing Cre recombinase (Cre) under *Pomc* promoter control were mated with mice carrying loxp-flanked p85 or p110 alleles. Immunohistochemistry for -endorphin was performed after ISHH for p85 (C) or p110 (D) in brain slices from targeted knockouts and littermate controls. *Green*, for.e.5956

Figure 2



Deletion of p85 or p110 in POMC neurons impacts body weight and metabolic parameters. A, Weekly weight gain in male *pik3r1 POMCKO* mice or *pik3CA POMCKO* mice and littermate controls on normal chow (P = 0.024, n = 7–11). B, Weight gain in female *pik3r1 POMCKO* mice or *pik3CA POMCKO* mice and littermate controls on normal chow (P = 0.42, n = 7–17). Food intake (C), ambulatory movement (D), heat production (E), O consumption (F), and respiratory

Figure 3

Altered body weight and adiposity in high-fat chow-fed (HFD) mice lacking p85 or p110 in POMC neurons. Weight gain in male (A) and female (B) *pik3r1 POMCKO* or *pik3CA POMCKO* mice and littermate controls were measured after introduction of high-fat chow at 3 months of age ($P = 0.030$ for *pik3CA POMCKO* males, $P = 0.015$ for *pik3r1 POMCKO* females and $P = 0.008$ for *pik3CA POMCKO* females; n = 7–8 male and 10–12 female cohorts). C–F, Fat and lean mass in 5-month-old *pik3r1 POMCKO*, *pik3CA POMCKO*, and littermate controls were analyzed by DEXA (*pik3r1 POMCKO* cohorts) or nuclear magnetic resonance (*pik3CA POMCKO* cohorts) (n = 6–12). G and H, Serum leptin levels were assessed by ELISA in 4-month-old *pik3r1 POMCKO*, *pik3CA POMCKO*, and littermate controls (n = 6–7). *, $P < 0.05$; **, $P < 0.01$.

Figure 4

Impaired insulin responsiveness in mice lacking p110 in POMC neurons A, Corticosterone levels (nanograms per milliliter) of *pik3r1 POMCKO*, *pik3CA POMCKO*, and littermate control mice (n = 6–8), while undergoing a glucose tolerance test. Tail vein blood samples were drawn 30 min into the test. B, Serum insulin levels were measured by ELISA after removal of food for 2 h in male *pik3r1 POMCKO*, *pik3CA POMCKO*, and Cre-negative littermate controls (n = 6–10). C, Blood glucose levels were measured by glucometer after removal of food for 2 h in male *pik3r1 POMCKO*, *pik3CA POMCKO*, and littermate controls (n = 6–8). Blood glucose response curve for glucose (D) and insulin (E) tolerance tests in *pik3r1 POMCKO*, *pik3CA POMCKO*, and littermate control mice (n = 6–8) is shown. Calculated areas under the curves are displayed adjacent to each. Tolerance tests were performed on male mice with 2 g glucose/kg or 1 U of insulin/kg body weight. For all of these experiments, the mice were 3 months of age; body weight of control and experimental mice did not differ significantly. Results are expressed as mean blood glucose concentration ± SEM. *, $P < 0.05$.

Figure 5

Corresponding changes in hepatic insulin sensitivity result from altered PI3K signaling in POMC neurons. A, Western blot analysis showed that phosphor-Akt (Ser-473) levels were dramatically reduced in *pik3CA POMCKO* hepatic tissue after insulin stimulation (5 U/kg ip, 10 min time point) compared with controls. Basal HGP (B) glucose infusion rate (C) and (D) suppression of HGP *in vivo* during hyperinsulinemic-euglycemic clamps in awake mice at 12–16 wk of age (*pik3r1 POMCKO* and controls, n = 8) are shown. E, PTP1B gene expression in male *pik3r1 POMCKO* and littermate controls after hyperinsulinemic-euglycemic clamp (n = 5–7). Body weights of control and experimental mice did not differ significantly. *, $P < 0.05$; **, $P < 0.01$. WT, Wild type; GINF, glucose infusion rate.

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