Rapid Publication

Insulin Activation of Phosphatidylinositol 3-Kinase in the Hypothalamic Arcuate Nucleus
A Key Mediator of Insulin-Induced Anorexia

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In peripheral tissues, insulin signaling involves activation of the insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase (PI3K) enzyme system. In the hypothalamus, insulin functions with leptin as an afferent adiposity signal important for the regulation of body fat stores and hepatic glucose metabolism. To test the hypothesis that hypothalamic insulin action involves intracellular PI3K signaling, we used histochemical and biochemical methods to determine the effect of insulin on hypothalamic IRS-PI3K activity. Here, we report that insulin induces tyrosine phosphorylation of the insulin receptor and IRS-1 and -2, increases binding of activated IRS-1 and -2 to the regulatory subunit of PI3K, and activates protein kinase B/Akt, a downstream target of PI3K. Using an immunohistochemical technique to detect PI 3,4,5-triphosphate, the main product of PI3K activity, we further demonstrate that in the arcuate nucleus, insulin-induced PI3K activity occurs preferentially within cells that contain IRS-2. Finally, we show that the food intake–lowering effects of insulin are reversed by intracerebroventricular infusion of either of two PI3K inhibitors at doses that have no independent feeding effects. These findings support the hypothesis that the IRS-PI3K pathway is a mediator of insulin action in the arcuate nucleus and, combined with recent evidence that leptin activates PI3K signaling in the hypothalamus, provide a plausible mechanism for neuronal cross-talk between insulin and leptin signaling.
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IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PIP_3, PI 3,4,5-triphosphate; PKB, protein kinase B.

In peripheral tissues (e.g., liver, muscle, and fat), the binding of insulin to its receptor stimulates autophosphorylation and activation of the insulin receptor intrinsic tyrosine kinase (1), leading to the recruitment and phosphorylation of members of the insulin receptor substrate (IRS) protein family (IRS1–4) (1). Tyrosine-phosphorylated IRS proteins, in turn, bind src homology 2 domain–containing signaling proteins, such as the p85 regulatory subunit of the type IA phosphatidylinositol 3-kinase (PI3K) (1), which activates the p110 catalytic subunit of PI3K. Activated PI3K rapidly mediates the phosphorylation of PI 4,5-biphosphate to PI 3,4,5-trisphoshpate (PIP_3) (2), a key signaling intermediate that recruits and activates downstream molecules, including serine-threonine kinases, tyrosine kinases, GTPases, and others (1). One key downstream target of PI3K is protein kinase B (PKB)/Akt, which is activated via serine and threonine phosphorylation (1).

Interestingly, homologues of the insulin receptor, IRS, PI3K, and PKB/Akt proteins are present in the nervous system of evolutionarily distant organisms such as *Caenorhabditis elegans* (3) and *Drosophila melanogaster* (4), in which they play an essential role in regulation of energy balance, reproductive fitness, and longevity. That an analogous role is played by this signaling pathway in mammalian brain (5) is suggested by the expression of these proteins in hypothalamic areas, such as the arcuate nucleus, that participate in the control of energy homeostasis. Recent evidence that the adipocyte hormone, leptin, activates IRS-PI3K signaling in rat hypothalamus (6,7) provides additional support for this hypothesis. Whether insulin signaling in mammalian brain also involves activation of the IRS-PI3K pathway is therefore an important unanswered question. Here, we use biochemical and histochemical methods to show that insulin activates hypothalamic IRS-PI3K signaling and localize this effect to arcuate nucleus neurons. Furthermore, we show that like leptin, hypothalamic signaling via PI3K is required for the effect of insulin to reduce food intake.

RESEARCH DESIGN AND METHODS

Animal studies. All procedures were performed in accordance with National Institutes of Health guidelines for the care and use of animals and were

approved by the Animal Care Committee at the University of Washington. Male Wistar rats (Charles River, Wilmington, MA) underwent third ventricular cannulation (8) 7–10 days before study. For biochemical studies, rats were fasted for 30 h, given an intracerebroventricular injection of insulin (10 mU) or vehicle (saline), and killed at intervals up to 60 min. The brain was rapidly removed, snap frozen, and a 100-mg wedge of mediobasal hypothalamus (defined caudally by the mammillary bodies, rostrally by the optic chiasm, laterally by the optic tract, and superiorly by the apex of the hypothalamic third ventricle) excised. For immunocytochemical analysis, the same protocol was followed except that 5 min after intracerebroventricular insulin (10 mU) and 15 min after a larger dose of peripheral insulin (5 units i.p.) or vehicle (intracerebroventricularly), animals were perfused via cardiac puncture with 60 ml of 0.1 mol/l PBS followed by 4% paraformaldehyde. These time points were chosen because they represent intervals over which robust biochemical PI3K activity is observed when insulin is given by each route. Perfused brains were removed and immersion fixed overnight in 4% paraformaldehyde followed by equilibration in 25% sucrose. Brains were snap frozen in liquid nitrogen–cooled isopentane and cryostat sectioned at 14 μm.

Biochemical analysis. Hypothalamic samples were lysed, homogenized, clarified, and normalized for protein content. One milligram of protein from each hypothalamus was immunoprecipitated overnight with anti–IRS-2 antibody (9) collected on protein A-Sepharose, washed, and assayed for PI3K activity as described (10). Lysates were also immunoprecipitated overnight with antibodies directed against the COOH-terminus of the insulin receptor, the p85 regulatory subunit of PI3K (Upstate Cell Signaling Resources, Waltham, MA), IRS-1, and IRS-2 (9). Immunoprecipitates were collected on protein A-Sepharose, washed, resolved by 7.5% SDS-PAGE, and immunoblotted with antiphosphotyrosine antibodies (4G10; Upstate Cell Signaling Resources); detection was by ^{125}I-protein A and exposure on a phosphorimager. Finally, similar lysates were resolved by 10% SDS-PAGE and immunoblotted with antibodies specific for the phosphorylated forms of PKB (Ser^{473} or Thr^{308}) (Cell Signaling Technology, Beverly, MA); detection was by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL) and film exposure.

Immunocytochemistry. Frozen sections were equilibrated in 0.1 mol/l PBS and blocked in 5% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA) and 2% BSA (Sigma, St. Louis, MO) in 0.1 mol/l PBS. All antibodies were diluted in this blocking buffer. Mouse anti-PIP_3 antibody (Echelon Biosciences, Salt Lake City, UT) (2,11) was used at a 1:100 dilution, and rabbit anti–IRS-2 (Upstate Cell Signaling Solutions, Waltham, MA) (9) at 1:300. Secondary antibodies (goat anti-mouse IgM conjugated to CY3 [Jackson Immunoresearch Laboratories] and goat anti-rabbit conjugated to Alexa488 [Molecular Probes, Eugene, OR]) were used at 1:200. Sections were mounted in polyvinylalcohol mounting medium and imaged under identical acquisition parameters on a Nikon Eclipse E600 upright epifluorescence microscope equipped with a Diagnostic Instruments Spot RT Color digital camera.

Food intake studies. Male Long Evans rats (Harlan, Indianapolis, IN) underwent third ventricular cannulation (8) 2 weeks before study and were habituated to regular handling. On the day of study, food was removed and rats received a single intracerebroventricular injection of the PI3K inhibitor LY294002 (LY; 1 nmol) (Calbiochem, San Diego, CA) or its vehicle 5 h before dark cycle onset. One hour after the LY injection, rats were weighed and received a single intracerebroventricular injection of insulin (4 mU, regular Iletin II purified pork; Eli Lilly, Indianapolis, IN) or its vehicle, generating four treatment groups: vehicle-vehicle, vehicle-insulin, LY-vehicle, and LY-insulin (n = 6–9/group). Food was returned 30 min before the onset of the dark cycle, and 4- and 24-h food intake and body weight change were recorded. Rats were rerandomized 3 days later and subjected to the same experimental protocol, except that the PI3K inhibitor wortmannin (0.01 nmol; Calbiochem) was substituted for LY294002 (n = 4–6/group).

Statistical analysis. Results are shown as means ± SEM for PI3K activity measurements and PKB/Akt phosphorylation. Statistical significance was calculated by two-tailed Student's t test between baseline sample and stimulated samples, with $P < 0.05$ considered significant. Food intake studies were analyzed by multivariate ANOVA with least significant difference posttest for differences between individual groups ($P < 0.05$ considered significant).

RESULTS

To investigate the hypothesis that insulin signaling in the hypothalamus involves activation of the IRS-PI3K cascade, we assayed IRS-2–associated PI3K activity in extracts of mediobasal hypothalamus obtained at various times after systemic insulin administration (5 units i.p.). We detected a significant stimulation of IRS-2–associated PI3K activity that peaked at 15–30 min and gradually declined thereafter

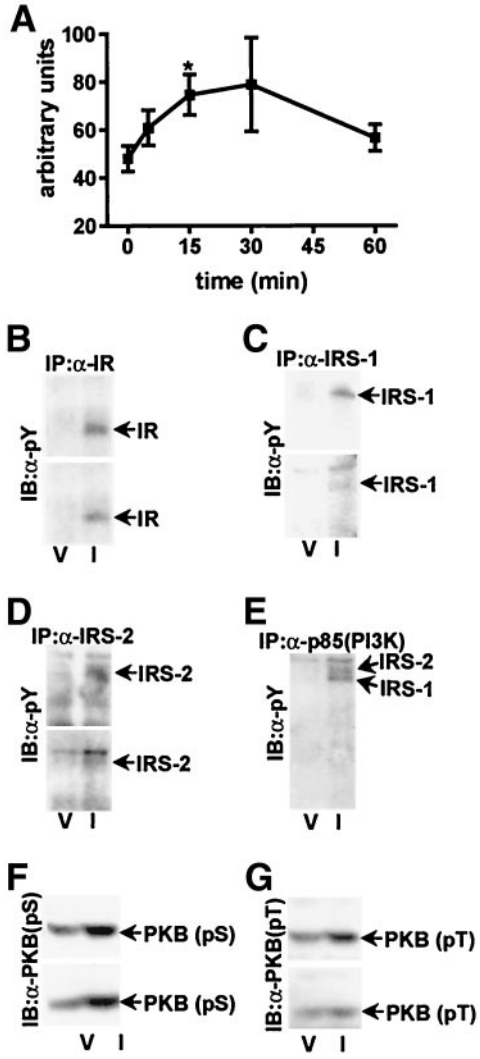

FIG. 1. Insulin activates PI3K signaling in mediobasal hypothalamus. *A*: Insulin (5 units) administered intraperitoneally induces activation of IRS-2–associated PI3K. Male Wistar rats were killed at indicated time points, and mediobasal hypothalamus was extracted and assayed for PI3K activity as described (10). *B*: Insulin (10 mU i.c.v. for 5 min) increases insulin receptor phosphotyrosine content in mediobasal hypothalamic extracts immunoprecipitated (IP) with anti-insulin receptor antibody (α-IR) and immunoblotted (IB) with an antiphosphotyrosine antibody (α-PY). Insulin treatment also increases IRS-1 (*C*) and IRS-2 (*D*) phosphotyrosine content, detected after immunoprecipitation with their respective antibodies. *E*: Similarly, insulin increases IRS-1 and -2 phosphotyrosine content and enhances the interaction of phosphorylated IRS-1 and -2 with the p85 subunit of PI3K in homogenates of rat mediobasal hypothalamus that were immunoprecipitated with an antibody to the p85 subunit of PI3K (α-p85). Insulin treatment (same homogenates) also enhances the phosphothreonine (*F*) and phosphoserine (*G*) content of PKB/AKT, as detected by immunoblotting with specific antibodies to threonine [α-PKB(pT)] and serine phosphorylated PKB [α-PKB(pS)].

(Fig. 1*A*), a time course that closely resembles the effect of leptin on hypothalamic PI3K (6). Similar effects were observed when a 500-fold–lower dose of insulin (10 mU) was injected directly into the third cerebral ventricle (not shown). Compared with vehicle-treated controls, intracerebroventricular insulin administration (10 mU) also induced tyrosine phosphorylation of the insulin receptor (Fig. 1*B*), as well as IRS-1 (Fig. 1*C*) and IRS-2 (Fig. 1*D*), and increased the association of tyrosine phosphorylated IRS-1 and -2 proteins with the p85 subunit of PI3K (Fig. 1*E*). Moreover, serine phosphorylation of PKB/Akt was

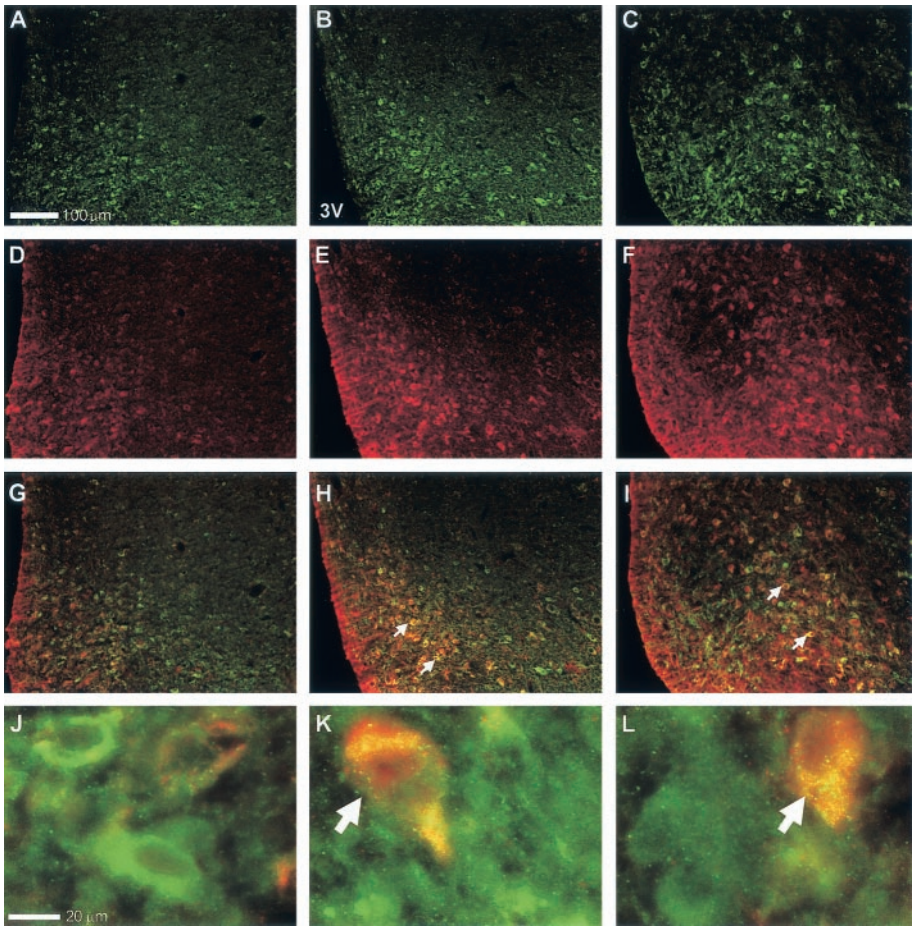

FIG. 2. Insulin enhances PIP_3 immunoreactivity primarily in IRS-2–expressing arcuate nucleus neurons (*A–I*: ×20 magnification, third ventricle at lower left of frame; *J–L*: ×100 view of ARC neurons). Rats were treated with intracerebroventricular vehicle (*A*, *D*, *G*, and *J*), intracerebroventricular insulin (10 mU, 5 min; *B*, *E*, *H*, and *K*), or peripheral insulin (5 units i.p.,15 min; *C*, *F*, *I*, and *L*); the sections of arcuate nucleus immunostained for IRS-2 (*A–C*) and PIP_3 (*D–F*); and the images merged at low (*G–I*) and high power (*J–L*). IRS-2 immunoreactivity was detected specifically in neurons of the arcuate nucleus with all treatments (*A–C*), and with no staining detected elsewhere. Insulin treatment, either intracerebroventricularly (*E*) or peripherally (*F*), induces increased PIP_3 immunoreactivity compared with vehicle treatment (*D*). Enhanced PIP_3 immunoreactivity occurs primarily in IRS-2–positive neurons (*G–I*). Especially at high magnification, PIP_3/IRS-2 double-positive neurons are identified in insulin-treated arcuate nucleus neurons (*K* and *L*), whereas few if any are observed in vehicle-treated rats (*J*). 3V, third cerebral ventricle.

increased 3.1-fold after intracerebroventricular insulin ($n = 4$, $P < 0.05$) (Fig. 1*F*) compared with vehicle, and threonine phosphorylated PKB/Akt was similarly increased (Fig. 1*G*). These findings confirm that insulin acts locally within the hypothalamus to activate the intracellular IRS-PI3K pathway and its downstream targets.

To identify hypothalamic neurons in which PI3K signaling is activated by insulin, we used an immunohistochemical approach to detect PIP_3 immunoreactivity (2,11). Following intracerebroventricular injection of a saline vehicle, relatively low-intensity PIP_3 immunofluorescence was detected in a small minority of cells in the arcuate nucleus of fasted rats (Fig. 2*D*). Five minutes after a single intracerebroventricular injection of insulin (10 mU), PIP_3 immunofluorescence markedly increased in many arcuate nucleus cells (Fig. 2*E* and *H*). In contrast, PIP_3 immunoreactivity was not increased in surrounding hypothalamic areas, including the ventromedial hypothalamic nucleus, zona incerta, and lateral hypothalamic area of insulin-treated rats (not shown). Systemic administration of insulin (5 units i.p., 15 min) similarly induced immunoreactive PIP_3 that was concentrated in the arcuate nucleus (Fig. 2*F* and *I*).

IRS-2, a key mediator of insulin action in peripheral tissues, is also synthesized in the hypothalamus (12). Moreover, mice lacking IRS-2 have increased body adiposity and impaired reproduction (13), a phenotype resembling that of mice with neuron-specific deletion of insulin receptors (14). We therefore hypothesized that IRS-2 expression would identify neurons with the potential to respond to insulin by activating PI3K. Consistent with published results using in situ hybridization to detect IRS-2 mRNA (12), we found that hypothalamic IRS-2 immunoreactivity was highly localized in neurons of the arcuate nucleus (Fig. 2*A–C*). Interestingly, IRS-2 immunoreactivity was present in the majority of arcuate nucleus cells that responded to insulin with induction of PIP_3 immunoreactivity, whether insulin was infused centrally (Fig. 2*H* and *K*) or peripherally (Fig. 2*I* and *L*), and this colocalization is readily detected at high magnification (×100; Fig. 2*K* and *L*). Future studies are warranted to determine the neuropeptide phenotype of arcuate neurons in which this occurs and whether PIP_3 immunoreactivity is induced in the many other brain areas that express insulin receptors.

To determine whether activation of PI3K by insulin is required for the ability of insulin to reduce food intake, we

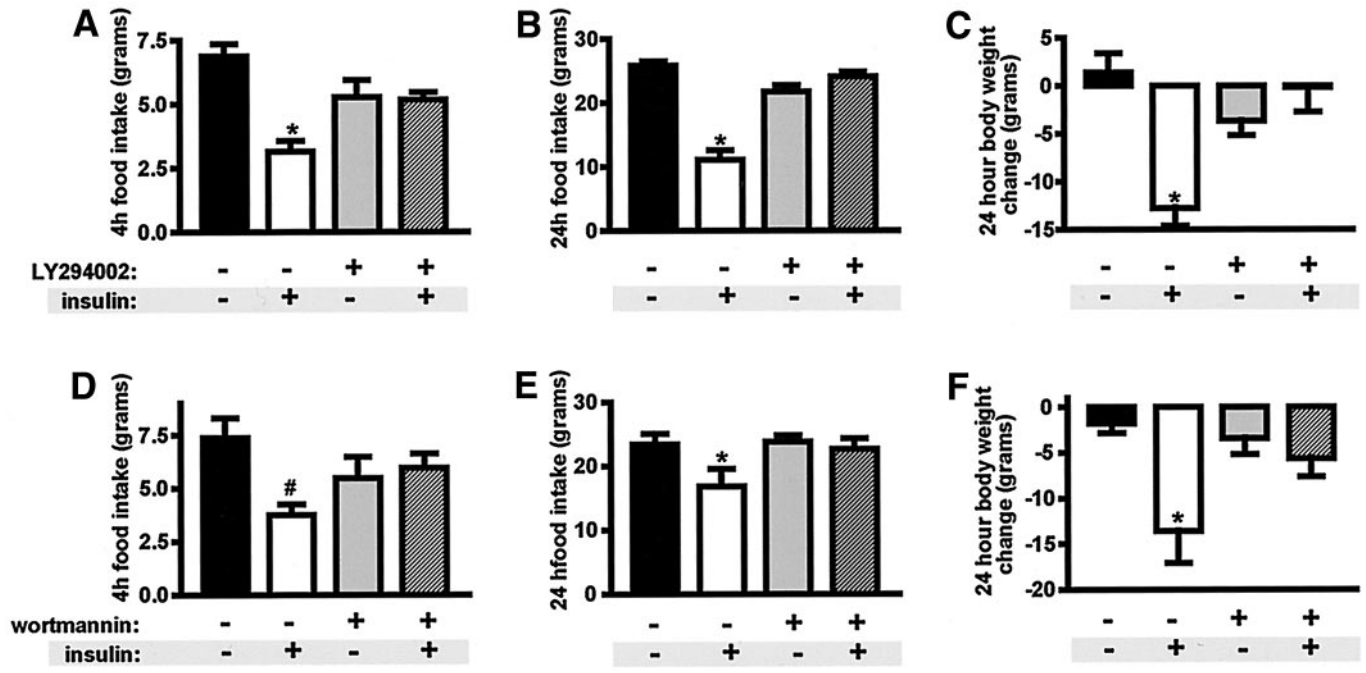

FIG. 3. Insulin-induced anorexia and weight loss are blocked by inhibitors of PI3K. Third ventricular administration of insulin (4 mU) 4 h before onset of the dark cycle (and 1 h after vehicle injection) significantly suppresses food intake during the first 4 h of the dark cycle (*A* and *D*) and for the full 24 h after injection (*B* and *E*). Body weight is similarly reduced at 24 h (*C* and *F*). Pretreatment with the PI3K inhibitors LY294002 (*A–C*; 1 nmol) or wortmannin (*D* and *E*; 0.01 nmol) 1 h before insulin administration prevents insulin-induced anorexia and weight loss (n = 6–9/group for *A–C* and n = 4–6/group for *D–F*, analyzed by multivariate ANOVA with least significant difference posttest). *$P < 0.05$ for vehicle-insulin group vs. vehicle-vehicle and PI3K inhibitor–insulin groups; #$P < 0.05$ for vehicle-insulin group vs. vehicle-vehicle group and $P < 0.10$ for vehicle-insulin group vs. wortmannin-insulin group.

infused inhibitors of PI3K into the third cerebral ventricle before injection of an anorectic dose of insulin. Consistent with previous reports (15), we found that intracerebroventricular insulin reduced food intake compared with vehicle treatment (Fig. 3*A*, *B*, *D*, and *E*), and this effect was accompanied by decreased body weight at 24 h after injection (Fig. 3*C* and *F*). Pretreament with either LY294002 (Fig. 3*A* and *B*) or wortmannin (Fig. 3*D* and *E*) blocked the ability of insulin to reduce food intake at both 4 and 24 h (Fig. 3*A*, *B*, *D*, and *E*) and to decrease body weight (Fig. 3*C* and *F*). The same doses of PI3K inhibitors had no effect, or slightly decreased food intake (P = NS), when given before vehicle instead of insulin (Fig. 3*A*, *B*, *D*, and *E*), indicating that they did not independently increase food intake. Because PI3K is the only enzyme known to be inhibited by both LY294002 and wortmannin, these data suggest that insulin's ability to reduce food intake is dependent on the activation of PI3K.

DISCUSSION

The arcuate nucleus is an important target for the action of hormones such as insulin and leptin in the control of both energy and glucose homeostasis (14,16–18). Receptors for both hormones are concentrated in this brain area (19), and, like leptin (20,21), insulin regulates the expression of arcuate nucleus neuropeptide genes, including those encoding neuropeptide Y (8) and pro-opiomelanocortin (15). Moreover, impaired hypothalamic signaling by either insulin (22) or leptin (23) activates arcuate nucleus neuropeptide Y neurons, increases food intake, and impairs glucose metabolism in peripheral tissues.

The hypothesis that insulin and leptin signal via a common biochemical pathway in the hypothalamic regulation of energy homeostasis provides a plausible mechanism to explain the many overlapping effects of these two hormones in the arcuate nucleus (5). Based on this hypothesis, we sought to determine whether the IRS-PI3K pathway mediates actions of both insulin and leptin in this brain area. In the current studies, we found that systemically administered insulin activates PI3K in mediobasal hypothalamus and that this effect is replicated by infusion of a low dose of insulin directly into the adjacent third ventricle. Furthermore, we found that IRS-2 immunoreactivity is concentrated in the arcuate nucleus and appears to identify a subset of cells that respond to insulin with PI3K activation. Concurrent biochemical and immunohistochemical data, therefore, support the hypothesis that insulin activates PI3K signaling selectively within neurons of the arcuate nucleus. To investigate the functional significance of this activation, we determined whether insulin-induced anorexia can be blocked by intracerebroventricular pretreatment with PI3K inhibitors. Our findings that insulin's inhibitory effect on food intake is blocked by either of two PI3K inhibitors supports an important physiological role for this intracellular signaling pathway. Together with recent evidence that leptin also signals through the hypothalamic IRS-PI3K pathway (6,7), these data suggest that intracellular PI3K signaling mediates responses downstream of receptors for insulin and leptin in hypothalamic neurons.

The hypothesis that PI3K mediates the hypothalamic effects of both insulin and leptin has potentially important implications for understanding how energy homeostasis becomes disrupted in obese states (5). Numerous obser-

vations that food intake is maintained at normal or increased levels despite elevated plasma concentrations of both hormones suggest that hypothalamic resistance to these adiposity signals may be commonplace in obese individuals (24). Based on growing evidence that impaired signaling through PI3K contributes to obesity-induced insulin resistance in peripheral tissues (25), the hypothesis that obesity similarly impairs neuronal signal transduction via PI3K provides a plausible mechanism to explain this phenomenon.

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