Insulin action in the brain contributes to glucose lowering during insulin treatment of diabetes

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Summary

To investigate the role of brain insulin action in the pathogenesis and treatment of diabetes, we asked whether neuronal insulin signaling is required for glucose-lowering during insulin treatment of diabetes. Hypothalamic signaling via the insulin receptor substrate-phosphatidylinositol 3-kinase (IRS-PI3K) pathway, a key intracellular mediator of insulin action, was reduced in rats with uncontrolled diabetes induced by streptozotocin (STZ-DM). Further, infusion of a PI3K inhibitor into the third cerebral ventricle of STZ-DM rats prior to peripheral insulin injection attenuated insulin-induced glucose lowering by ~35%–40% in both acute and chronic insulin treatment paradigms. Conversely, increased PI3K signaling induced by hypothalamic overexpression of either IRS-2 or protein kinase B (PKB, a key downstream mediator of PI3K action) enhanced the glycemic response to insulin by ~2-fold in STZ-DM rats. We conclude that hypothalamic insulin signaling via the IRS-PI3K pathway is a key determinant of the response to insulin in the management of uncontrolled diabetes.

Introduction

In addition to its effects in peripheral tissues, the pancreatic hormone insulin communicates information regarding the sufficiency of energy stores to brain areas involved in the control of energy homeostasis and glucose metabolism (Flier, 2004; Obici and Rossetti, 2003; Schwartz and Porte, 2005). Acting through its receptor in hypothalamic areas such as the arcuate nucleus (ARC) (Schwartz et al., 1992a), insulin promotes negative energy balance (by reducing food intake) (Woods et al., 1979) and reduces blood-borne concentrations of nutrients such as glucose (by inhibiting endogenous glucose production) (Obici et al., 2002c). A physiological role for these actions of insulin is suggested by the obese, insulin-resistant phenotype of mice lacking neuronal insulin receptors (Bruning et al., 2000), and by evidence that in rats, reduced hypothalamic insulin signaling increases both food intake and hepatic glucose production (Obici et al., 2002a). Similar effects are seen in mice in which IRS-2, an intracellular mediator of insulin signaling, is deleted from both hypothalamic neurons and pancreatic β cells using Cre-loxP technology (Kubota et al., 2004; Lin et al., 2004). In addition, the ability of a physiological increase of plasma insulin levels to suppress hepatic glucose production requires its action in mediobasal hypothalamus in normal rats (Pocai et al., 2005a). These findings raise the possibility that in individuals with diabetes, the ability of insulin to normalize elevated blood glucose levels depends in part on its action in the brain. Investigating this hypothesis provides the central focus for the current work.

As in peripheral tissues, neuronal insulin action involves the intracellular IRS-PI3K signal transduction pathway. Thus, hypothalamic IRS-PI3K signaling increases following either intracerebroventricular (icv) or systemic insulin administration (Niswender et al., 2003), and the inhibitory effect of icv insulin on both food intake (Niswender et al., 2003) and hepatic glucose production (Obici et al., 2002b) can be blocked by icv pretreatment with a PI3K inhibitor. We therefore sought to determine whether hypothalamic PI3K signaling is reduced in rats with untreated insulin-deficient diabetes mellitus induced by the β cell toxin, streptozotocin (referred to here as "uncontrolled diabetes" or "STZ diabetes") (Booth, 1972) and if so, whether hypothalamic signaling via this is pathway plays an essential role in the glycemic response to insulin.

Results

As previously reported (Havel et al., 2000; Qu et al., 2001), STZ administration to male Sprague-Dawley rats induced marked hyperglycemia (Figure 1A) and hypoinsulinemia (STZ-DM, 36 ± 4 pmol/L versus Non-Diabetic, 231 ± 21 pmol/L, $p < 0.0001$, $n = 8$) that was associated with increased hypothalamic *Npy* and *Agrp* (Figures 1B and 1D) and reduced pro-opiomelanocortin (*Pomc*) mRNA levels (Figure 1D). The content of serine-phosphorylated protein kinase B (PKB-PO_4, a marker of PI3K activity) in extracts of mediobasal hypothalamus was reduced by 36% in STZ- compared to vehicle-treated rats as determined by ELISA (Figure 1E), a finding confirmed by immunoblot analysis (data not shown). Twenty minutes after central insulin administration (5 mU icv), hypothalamic serine-PKB-PO_4 levels were markedly increased in STZ-DM rats studied at both early (by 2.5-fold over vehicle on day 8; $p < 0.001$; $n = 4–6$) and later time points (by 2.8-fold on day 21; $p < 0.001$; $n = 5-6$; Figure 1F)

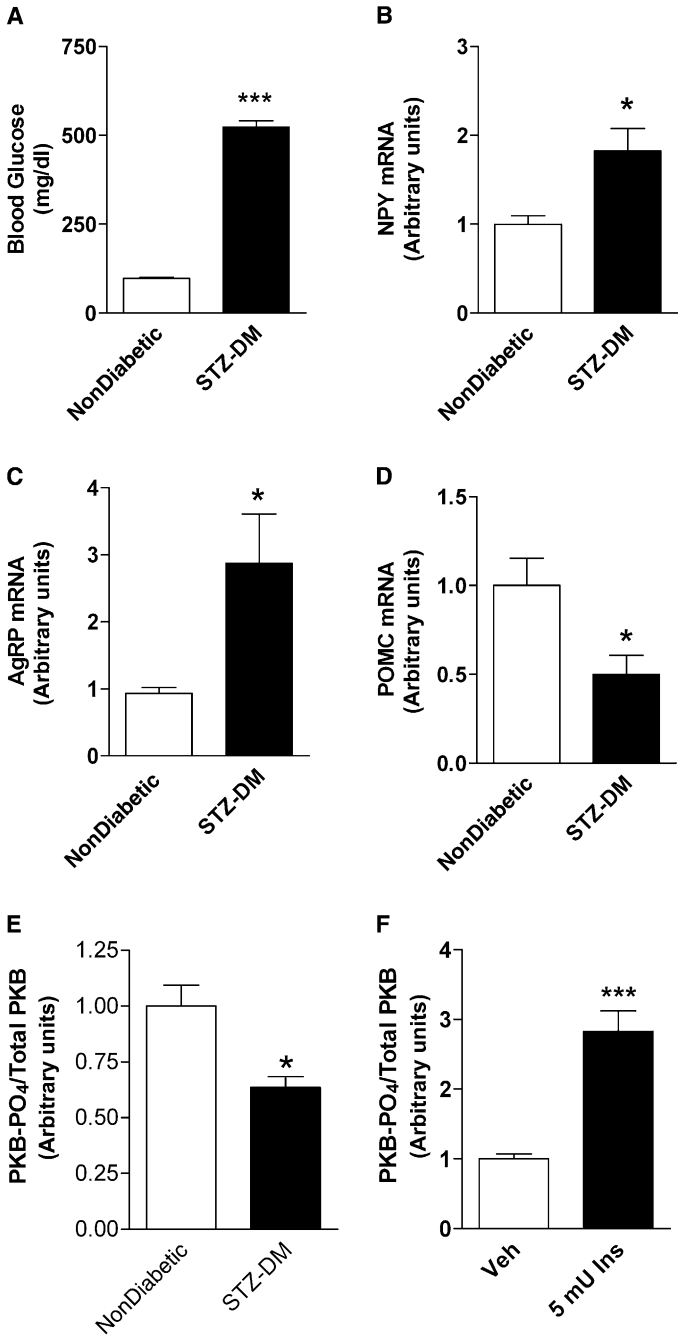

Figure 1. Effects of STZ-DM on hypothalamic PI3K signaling and neuropeptide gene expression

Eight days after sc injection of vehicle or STZ, rats were sacrificed for measurement of (**A**) blood glucose levels, (**B–D**) hypothalamic neuropeptide gene expression, or (**E**) hypothalamic levels of phsophoSER473-PKB and total PKB levels by ELISA. (**F**) Hypothalamic phsophoSER473-PKB levels were measured 20 min after vehicle or insulin administration (5 mU icv) to rats with STZ-DM. Data are mean ± SEM, n = 6–8/group. *, $p < 0.05$; ***, $p < 0.001$ versus control.

after diabetes onset. Insulin stimulation of PI3K also induces tyrosine phosphorylation of threonine$_{305}$ of PKB, and hypothalamic content of threonine-phosphorylated PKB was increased by insulin treatment in STZ-DM rats (Figure S1 in the Supplemental Data available with this article online). Signaling via the IRS-PI3K pathway is therefore reduced in the mediobasal hypothalamus of rats with STZ-DM, and this effect is rapidly reversed with insulin treatment.

To determine whether increased hypothalamic IRS-PI3K signaling is required for intact glucose lowering during insulin treatment of diabetes, two consecutive pretreatment injections of the PI3K inhibitor LY294002 (LY; 1 nmol icv, a dose that blocks the anorexic response to icv insulin (Niswender et al., 2003)) or its vehicle were administered (at −12 hr and −1 hr) to rats with STZ-DM prior to a systemic insulin injection (2 U/kg ip). Compared to STZ-DM rats receiving icv vehicle, icv pretreatment with LY attenuated the glycemic effect of systemically-administered insulin by 35% (Inverse AUC$_{Glucose}$: 16324 ± 4541 versus 25756 ± 757 mg/dl/120 min, $p < 0.05$, n = 7–11; Figures 2A and 2B). This effect cannot be attributed to leakage of the PI3K inhibitor into the periphery, since ip administration of the same LY dose did not alter insulin's glucose-lowering effect (Figures 2C and 2D).

To investigate whether hypothalamic IRS-PI3K signaling is a determinant of insulin requirements during chronic insulin treatment of diabetes, STZ-DM rats underwent sc implantation of an osmotic minipump that delivered either LY (200 ng/0.50 μl/h) or vehicle continuously into the third cerebral ventricle. Three days later, daily treatment with either a long-acting insulin preparation (insulin glargine, 7 mU/kg sc) or saline vehicle was commenced. Whereas icv LY infusion had no effect on the hyperglycemia induced by STZ in the absence of insulin treatment (Figure 2E), the glycemic response to daily insulin treatment was attenuated by 38% in animals receiving continuous icv infusion of LY (Figure 2F). Hypothalamic insulin signaling via IRS-PI3K, therefore, appears to be necessary for effective glucose lowering by insulin in the daily management of diabetes. Since the inhibitory action of LY is not 100% specific for PI3K, the possibility remains that inhibition of other neuronal enzyme systems contributed to this outcome. One way to address this issue is to determine whether an increase of hypothalamic signaling via IRS-PI3K induces an effect opposite to that of icv LY infusion in our model.

To accomplish this, we directed adenovirus encoding either IRS-2 (AdV-IRS2) or a reporter gene (AdV-lacZ or AdV-GFP) to the ARC of STZ-DM rats using an established stereotaxic microinjection technique (Morton et al., 2005, 2003). Successful localization of adenovirus expression to the area of the ARC (Figure 3A) and induction of IRS-2 expression in this brain area was confirmed in a subset of animals (Figure 3B). Four days following AdV delivery, STZ was given to both groups and, as expected, blood glucose levels rose steadily. However, both the onset of STZ-induced hyperglycemia and timing of peak blood glucose values were delayed in rats receiving AdV-IRS2 compared to AdV-lacZ (Figure 3C), despite no difference in either food intake (Figure 3D) or body weight (Δbody weight for the 5-day period following STZ-treatment; AdV-IRS2, + 0.9 ± 4.5 versus AdV-lacZ, –1.1 ± 8.7 g, n = 5-8) between groups. Nevertheless, blood glucose levels in STZ-DM rats receiving Ad-IRS2 eventually rose to values comparable to those of animals treated with AdV-lacZ.

To explain this outcome, we hypothesized that ARC-directed IRS-2 gene therapy increased insulin sensitivity in peripheral tissues and thereby slowed the rise of plasma glucose induced by falling insulin levels. Once pronounced STZ-induced hypoinsulinemia was established, however, we reasoned that the insulin-sensitizing effect conferred by increased hypothalamic PI3K

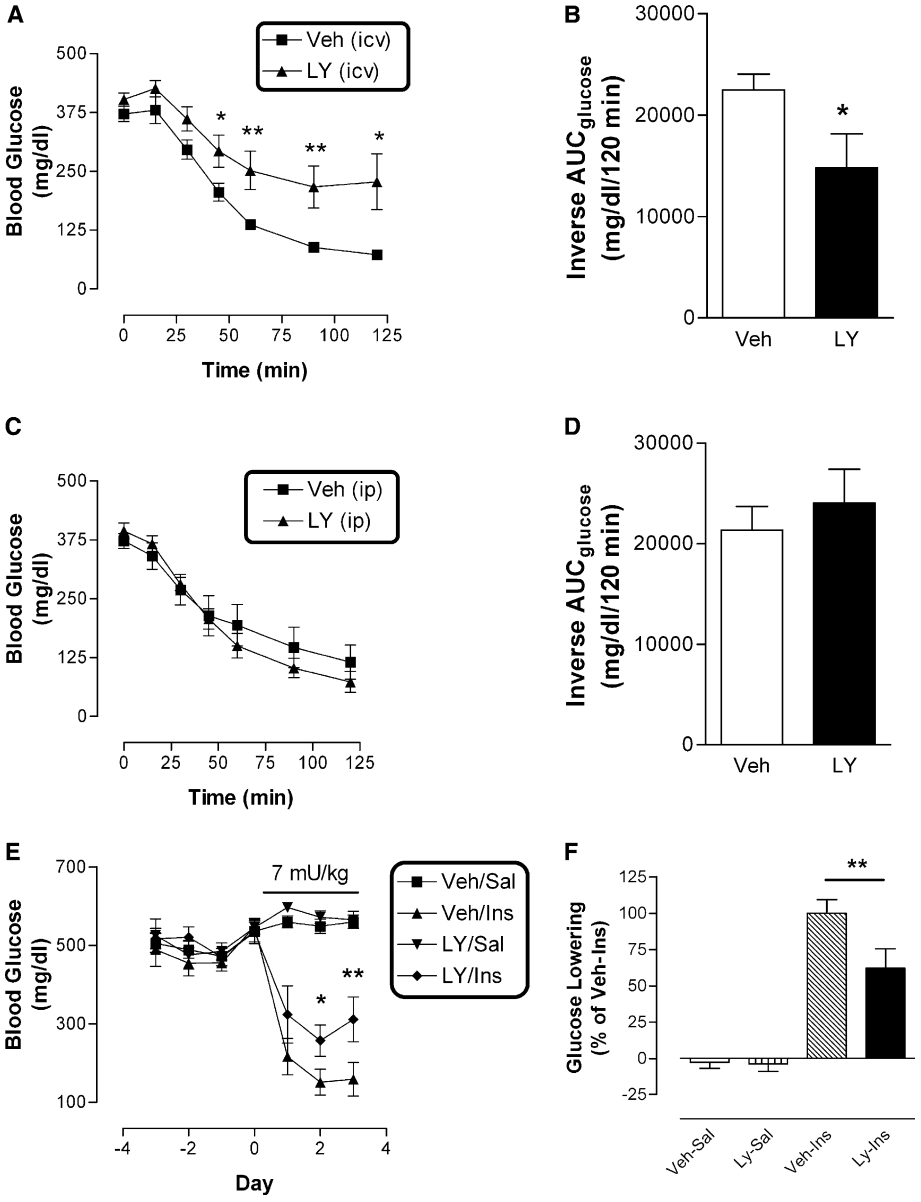

Figure 2. Attenuation of the glycemic response to insulin in STZ-DM rats following icv pretreatment with a PI3K inhibitor

Eight days following induction of STZ-DM, rats received two consecutive icv injections of either LY294002 (LY) or vehicle at 12 hr and 1 hr prior to being challenged with insulin (2 U/kg ip).

A) Blood glucose was determined every 15–30 min over 2 hr and (**B**) the inverse integrated area under the curve (Inverse AUC glucose) was determined. To control for leakage of LY into the bloodstream, the response to insulin was measured (**C** and **D**) in separate groups of STZ-DM rats that received an ip injection of the same dose of LY or vehicle. Data represent the mean ± SEM, n = 7–11/group. *, $p < 0.05$; **, $p < 0.01$; Ly versus Veh. (**E** and **F**) Effect of continuous icv delivery of the PI3K inhibitor LY294002 on the ability of daily administration of a long-acting insulin analog, insulin glargine, to reduce blood glucose levels in STZ-DM rats. Insulin/Veh treatment began on day 0 (day 14, post STZ treatment). Data are mean ± SEM, n = 7–9/group. *, $p < 0.05$; **, Ly/Ins versus Veh/Ins.

signaling would no longer be detectable. To test this hypothesis, we measured the ability of systemic insulin to lower blood glucose in STZ-DM rats after hypothalamic gene therapy with either IRS-2 or control adenovirus. Because of difficulties inherent in detecting a heightened glucose-lowering response to systemic insulin treatment (2 U/kg ip) in animals with normal insulin sensitivity, we chose to study rats with STZ-DM of sufficient duration (18 days) to become insulin resistant due to chronic insulin deficiency (Bevilacqua et al., 1985; Maegawa et al., 1986). As expected, the glucose-lowering effect of insulin in animals on day 18 was reduced relative to rats with STZ-DM of shorter duration (Figures 3E and 3F), compatible with the induction of insulin resistance, and this response to insulin was increased >2-fold by ARC-directed IRS-2 gene therapy compared to STZ-DM rats receiving a control adenovirus (Figures 3G and 3H; Inverse $AUC_{Glucose}$: 12562 ± 2301 versus 5625 ± 1201 mg/dl/120 min. $p < 0.05$, n = 5–8). Increased hypothalamic IRS-2 expression, therefore, strongly enhances the response of diabetic rats to systemic insulin treatment of diabetes.

Because IRS-2 links the insulin receptor to multiple signal transduction pathways (e.g., MAP kinase, in addition to PI3K), we sought to provide an additional test of the hypothesis that increased hypothalamic signaling via the IRS-PI3K pathway augments the glucose-lowering response to insulin in rats with uncontrolled diabetes. We therefore repeated the above study, substituting an adenovirus expressing a constitutively active mutant of PKB (AdV-CA) for AdV-IRS2. As predicted, we found that the ability of systemic insulin to lower blood glucose in rats with STZ-DM was augmented by nearly 2-fold by ARC directed microinjection of AdV-CA relative to control adenovirus (AdV-Luc) (Figures 3I and 3J). In a separate group of animals with STZ-DM, we replicated the effect of ARC-directed gene therapy with AdV-IRS2 to potentiate insulin-induced glucose lowering by ~2-fold (data not shown).

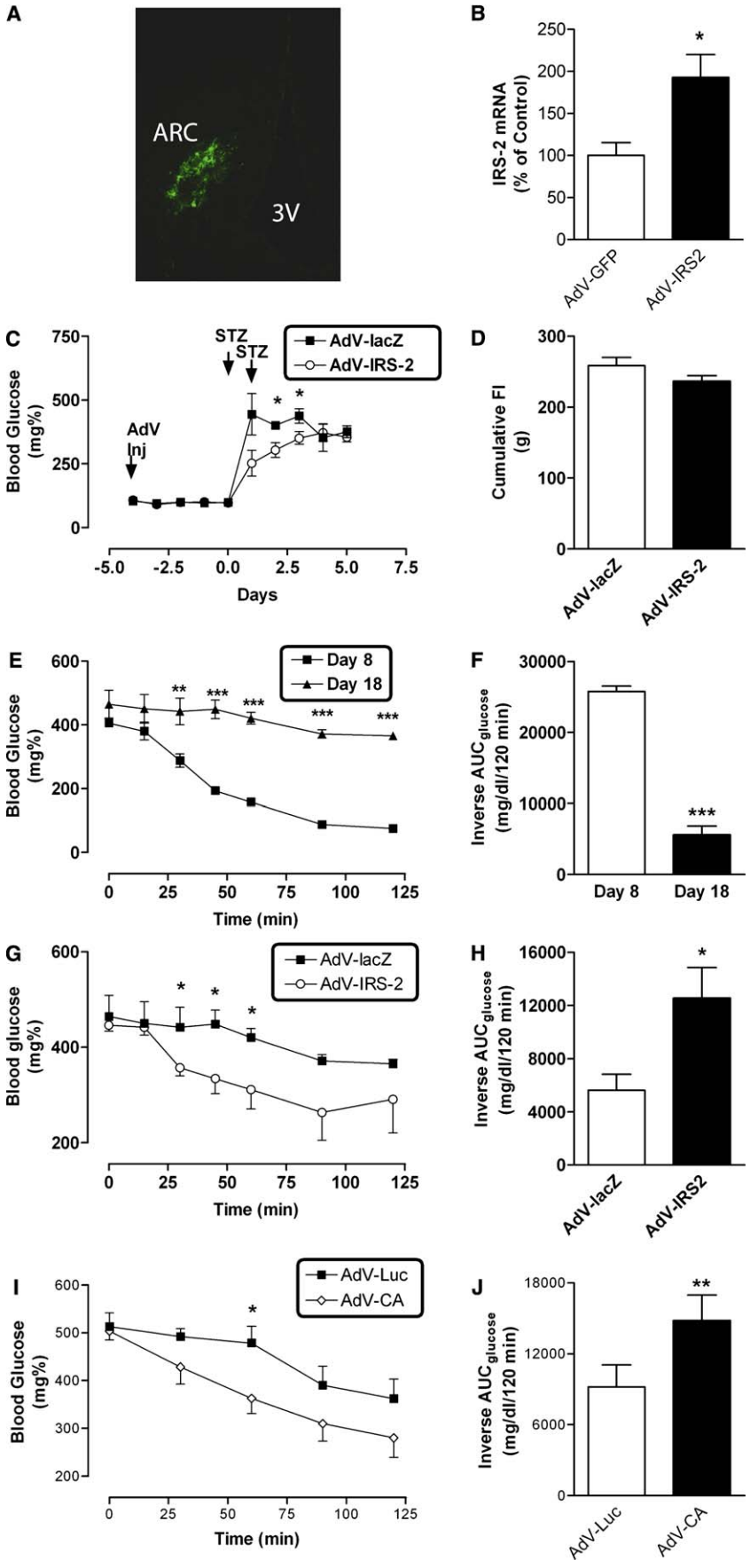

Figure 3. Effect of duration of STZ-DM on glucose lowering by insulin and hypothalamic adenoviral gene therapy on food intake and blood glucose levels in STZ-DM rats

Expression of GFP (**A**) and IRS-2 (**B**) in mediobasal hypothalamus following ARC-directed adenovirus microinjection. (**C**) Time course of changes in blood glucose or 5 day cumulative food intake (**D**) in STZ-DM rats receiving ARC-directed IRS-2 gene therapy or a control adenovirus (AdV-lacZ). (**E** and **F**) Glucose-lowering effect of insulin (2 U/kg ip) measured 8 d and 18 d following STZ administration to male Sprague Dawley rats. (**G** and **H**) Glucose-lowering effect of insulin (2 U/kg ip) measured 18 d following STZ administration, in STZ-DM rats receiving adenoviral gene therapy with AdV-IRS2 or reporter virus (AdV-lacZ). (**I** and **J**) A similar enhancement of insulin's ability to lower blood glucose was observed in STZ-DM rats receiving gene therapy with AdV-CA compared to reporter virus (AdV- Luc). Data represent the mean ± SEM, n = 5–9/group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus Con.

Discussion

Until recently, the effects of insulin on glucose metabolism were thought to be mediated solely by its actions on peripheral tissues. However, mounting evidence in nondiabetic animal models has challenged this concept, revealing an important role for neuronal insulin action in glucose homeostasis. For example, the effect of third ventricular insulin administration to increase the ability of circulating insulin to inhibit hepatic glucose production in rats is well documented (Obici et al., 2002b), as is evidence that reduced hypothalamic insulin action causes hepatic insulin resistance (Obici et al., 2002a, 2002b). Additional support stems from studies in which insulin receptors were restored selectively to the brain, liver and pancreas of mice that otherwise lack this receptor using a transgenic approach (Okamoto et al., 2004). Collectively, these findings raise the possibility that treatment of uncontrolled diabetes depends upon intact neuronal insulin signaling and, therefore, that brain insulin action is a determinant of insulin requirements among individuals with diabetes.

Our current studies support both of these predictions. We found that IRS-PI3K signaling is reduced in the mediobasal hypothalamus of rats with STZ-DM, that this reduction is readily reversed by insulin treatment, and that infusion of an inhibitor of PI3K signaling into the 3rd cerebral ventricle attenuates the glycemic response of diabetic animals to systemic insulin treatment. Thus, hypothalamic insulin signaling is required for an intact glycemic response to insulin treatment of diabetes. Conversely, blood glucose lowering by systemic insulin treatment was greatly enhanced by increasing signaling upstream (IRS-2) of PI3K in the mediobasal hypothalamus of animals with STZ-DM. Surprisingly, we did not detect an increase of insulin-induced serine-phosphorylated PKB content in diabetic rat hypothalamus following Ad-IRS2 gene therapy (data not shown). We therefore hypothesized that our assay of mediobasal hypothalamic extracts lacks the sensitivity required to detect changes of PI3K signaling that occur in the relatively small proportion of cells transduced by adenovirus in vivo. To further investigate whether increased IRS-PI3K signaling mediates the response to a localized increase in hypothalamic expression of IRS2, we injected adenovirus encoding a constitutively active PKB mutant into the area of the ARC of STZ-DM rats. The augmentation of insulin-induced glucose lowering resulting from this intervention mimicked that seen with hypothalamic IRS-2 gene therapy. This finding suggests both that the response to Ad-IRS-2 involves signal transduction via PKB and that increased hypothalamic signaling either upstream (IRS-2) or downstream (PKB) of PI3K is sufficient to enhance insulin-induced glucose lowering in diabetic rats. These findings collectively implicate hypothalamic insulin signaling via the IRS-PI3K pathway as a key determinant of the glycemic response to insulin in animals with uncontrolled diabetes. The sensitivity of the brain to insulin may therefore represent a previously unrecognized determinant of insulin requirements in patients with diabetes.

Among neuronal subsets with the potential to mediate the central action of insulin on glucose metabolism, NPY/Agrp neurons in the ARC are of particular interest. In addition to exerting potent orexigenic effects, central administration of NPY sharply decreases hepatic insulin sensitivity (Marks and Waite, 1997; van den Hoek et al., 2004) whereas both hyperphagia and hyperglycemia induced by STZ-DM are attenuated in mice with genetic NPY deficiency (Sindelar et al., 2002). Thus, both behavioral and glycemic manifestations of uncontrolled diabetes appear to depend in part on NPY signaling. Since insulin inhibits hypothalamic NPY gene expression (Schwartz et al., 1992b), it is temping to speculate that the effect of insulin deficiency to activate hypothalamic NPY neurons contributes to hyperglycemia, as well as to hyperphagia, characteristic of uncontrolled diabetes. According to this model, effective insulin treatment of uncontrolled diabetes is predicted to depend upon inhibition of these key hypothalamic neurons, and additional studies are warranted to investigate this hypothesis.

While the efferent mechanism coupling hypothalamic insulin signaling to changes of peripheral glucose metabolism remains to be fully elucidated, recent findings suggest that activation of K_{ATP} channels in mediobasal hypothalamic neurons is required for this effect (Pocai et al., 2005a). In this regard, the hypothalamic response to insulin closely resembles the effect of increased hypothalamic long-chain fatty acyl Co-A signaling to reduce hepatic glucose production (Pocai et al., 2005b). This response to both insulin and fatty acyl Co-A involves communication between hypothalamic neurons and hindbrain areas controlling parasympathetic outflow to the liver via the vagus nerve.

In summary, we report that insulin signaling via the hypothalamic IRS-PI3K pathway is a determinant of the glycemic response of animals with uncontrolled diabetes to systemic insulin treatment. Therapeutic strategies that target neuronal insulin signal transduction molecules may therefore prove beneficial in the management of diabetes in humans.

Experimental procedures

Animals

All experimental protocols were approved by the University of Washington Institutional Animal Care and Use Committee. Adult male Sprague Dawley rats (Charles River, Wilmington, MA) weighing 250–350 g were individually housed, fed a standard commercial diet (LabDiet, Richmond, IN) and provided with water *ad libitum*. Animals were maintained on a 12h/12h light/dark cycle with lights on at 0600h. Uncontrolled diabetes was induced with two consecutive daily subcutaneous (sc) doses of freshly prepared streptozotocin (40 mg/kg dissolved in ice-cold 0.1 M sodium citrate, pH 4.5), and blood glucose levels (from tail capillary samples), body weight and food intake were measured daily. Cannulation of the third cerebral ventricle was performed as previously described (Schwartz et al., 1992b). After a one-week recovery period, correct cannula placement was assessed by measuring the drinking response to icv injection of angiotensin II (AGII; 10 μg; American peptide, Sunnyvale, CA) diluted saline (injection volume: 1 μl) (Schwartz et al., 1992b).

Study protocols: Effect of icv LY294002 on the response to insulin

Acute study

To investigate whether hypothalamic PI3K signaling is required for the glycemic response to insulin, two groups of rats outfitted with an indwelling 3rd venricular cannula were studied 1 wk after STZ administration. Each animal received an icv injection of either LY294002 (1 nmol) or its vehicle both 12- and 1 hr prior to a systemic insulin challenge. The acute glycemic response to insulin was determined by measuring blood glucose levels at 15–30 min intervals for 2 hr after the administration of insulin (2 U/kg ip). While this approach to the measurement of insulin action can be confounded by induction of counter-regulatory responses in the event of hypoglycemia, the current studies were limited to hyperglycemic STZ-diabetic rats that did not develop hypoglycemia during insulin treatment.

Chronic study

One week after STZ administration to two groups of rats (n = 14-18/group), an osmotic minipump (Alzet minpumps, DURECT Corporation, Cupertino, CA) that delivered either LY294002 (200 ng/0.5 μl/h) or vehicle was implanted subcutaneously and connected to an indwelling 3rd ventricular cannula. After

a 6 day recovery period (14-d following STZ treatment), one-half of the animals in each group received saline control sc, while the other half received the long acting insulin preparation, insulin glargine (Lantus, Aventis Pharmaceuticals, Inc., Germany; 7 mU/kg sc). Blood glucose, food intake and body weight were measured daily for 3 d.

Response to adenoviral gene therapy

Adenoviruses (AdV) expressing either a reporter gene (lacZ, GFP or Luciferase), IRS-2, or a constitutively active PKB mutant (AdV-CA) were generated and purified as previously described (Lingohr et al., 2002). Rats were placed in a stereotaxic frame (Cartesian Research, Inc., Sandy, OR) under isoflurane anesthesia. The ARC was targeted bilaterally by performing two unilateral injections using a WPI UMPII Pump Adaptor (WPI, Sarasota, FL) and a 28-g syringe system (Hamilton Syringes, Reno, NV) directed to stereotaxic coordinates 2.8 mm posterior to bregma, ± 0.30 mm lateral to the midline, and 10.5 mm below the surface of the skull (Morton et al., 2005; Morton et al., 2003). Adenovirus (2×10^{12} pfu/ml) was infused at a rate of 100 nl/min for 5 min (500 nl/injection site). Both the anatomical distribution of and successful expression of protein products of the encoded genes have been extensively characterized and adenoviral delivery of reporter genes to the area of the ARC does not influence daily food intake or body weight in normal rats (Morton et al., 2003). Four days following ARC-directed gene therapy, STZ was administered and blood glucose, food intake and body weight were measured daily. On day 18 following the induction of diabetes, the acute glycemic response to an insulin challenge (2 U/kg ip) was determined.

Effect of STZ-DM on hypothalamic gene expression and PI3K signaling

Eight days following STZ injection, animals (n = 8–16/group) were sacrificed at 0900–1000 by decapitation and trunk blood was collected for determination of hormone levels. A wedge of mediobasal hypothalamus (defined caudally by the mamillary bodies; rostrally by the optic chiasm; laterally by the optic tract; and superiorly by the apex of the hypothalamic third ventricle) was rapidly dissected and frozen for subsequent biochemical analysis or mRNA determination as previously described (Morton et al., 2003). In a subset of these studies, icv saline or insulin (5 mU in 2 μl of saline) was administered into the third ventricle on either day 8 or 21 following the induction of STZ-DM. Rats were fasted for 6 hr prior to icv injection and were sacrificed 20 min later for removal of mediobasal hypothalamus and trunk blood as above.

Biochemical analysis

Hypothalamic samples were lysed, homogenized and clarified, and protein content determined. Thirty μg of total 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 cheimiluminescence (Pierce Biotechnology, Rockford, IL) and film exposure. Total and phosphorylated levels of PKB (Ser^{473}) were also determined with a commercially available ELISA (Biosource International, Camarillo, CA).

mRNA quantitation

RNA from hypothalamic tissue was isolated and underwent RT-PCR quantification as previously described (Morton et al., 2003). Primers were designed to span an exon/intron boundary were optimized for mRNA encoding *Npy*, *Agrp*, *Pomc*, *irs-2* and *Gapdh*. Primer sequences are listed: NPY: fwd 5′-accaggcagagatatggcaaga-3′; rev 5′-ggacattttctgtgctttctctcatta-3′; Agrp: fwd 5′-agggcatcagaaggcctgaccagg-3′; rev 5′-cattgaagaagcggcagtagcacgt-3′; POMC: fwd 5′-cgctcctactctatggagcactt-3′; rev 5′-gtcggtagactcttcccagcat-3′; IRS-2: fwd 5′-tcaccccagtgtccccat-3′; rev-5′tgctgttgccttcactgctt-3′; GAPDH: fwd 5′-aacgacccttcattgac-3′; rev 5′-tccacgacatactcagcac-3′.

Blood and plasma assays

Glucose levels were determined using a hand-held glucometer (Accu-Check, Roche, Diagnostics, IN) on blood obtained from tail capillary samples. Plasma immunoreactive insulin levels were determined by ELISA (Crystal Chem, Inc., Chicago, IL).

Statistical analyses

Data are presented as mean ± SEM. For multiple group comparisons, statistical significance was determined by one-way ANOVA using a Newman-Keuls *post-hoc* test to assess differences between groups. For two-group comparisons, an unpaired, two-tailed, Student's t test was employed. A p value < 0.05 was considered statistically significant.

Supplemental data

Supplemental Data include one figure and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/1/67/DC1/.

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