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Brain Insulin Controls Adipose Tissue Lipolysis and Lipogenesis

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SUMMARY

White adipose tissue (WAT) dysfunction plays a key role in the pathogenesis of type 2 diabetes (DM2). Unrestrained WAT lipolysis results in increased fatty acid release, leading to insulin resistance and lipotoxicity, while impaired de novo lipogenesis in WAT decreases the synthesis of insulin-sensitizing fatty acid species like palmitoleate. Here, we show that insulin infused into the mediobasal hypothalamus (MBH) of Sprague-Dawley rats increases WAT lipogenic protein expression, inactivates hormone-sensitive lipase (Hsl), and suppresses lipolysis. Conversely, mice that lack the neuronal insulin receptor exhibit unrestrained lipolysis and decreased de novo lipogenesis in WAT. Thus, brain and, in particular, hypothalamic insulin action play a pivotal role in WAT functionality.

INTRODUCTION

Adipose tissue functionality plays a critical role in normal glucose and lipid homeostasis. One of the major functions of white adipose tissue (WAT) is the release of nonesterified fatty acids (NEFAs) into the circulation during fasting and other energy-demanding states, such as exercise. Unrestrained lipolysis, which leads to increased circulating fatty acids in the absorptive state, has been linked to muscle (Bergman and Ader, 2000; Boden, 2006) and liver (Boden et al., 1994) insulin resistance as well as hepatic steatosis (Ginsberg et al., 2006). Total fatty acid flux increases with adiposity, consistent with unrestrained lipolysis (Mittendorfer et al., 2009). Recently, it has been demonstrated that the insulin-sensitizing fatty acid palmitoleate, termed a lipokine, is mainly produced in WAT via de novo lipogenesis (Cao et al., 2008). In obese humans, there is evidence for reduced WAT de novo lipogenesis (Diraison et al., 2002; Roberts et al., 2009), which further supports the concept that WAT represents a key link between obesity and the insulin-resistant state. However, the pathogenesis of adipose tissue dysfunction is still poorly understood.

Humans with insulin receptor mutations exhibit severe lipodystrophy and elevated circulating fatty acids, which highlights the pivotal role that insulin signaling plays in the maintenance of WAT functionality (Hegele, 2003). Insulin is considered the major antilipolytic hormone. Its antilipolytic effects are thought to be exclusively mediated through insulin receptors expressed on adipocytes (Degerman et al., 2003). Cyclic AMP (cAMP) signaling represents the principal prolipolytic pathway in WAT, which is chiefly regulated by the sympathetic nervous system (SNS). However, it is presently unknown whether insulin regulates WAT lipolysis by inhibiting sympathetic outflow through brain effects, and if so, in which anatomical brain structure. The proposition that insulin action in the brain could regulate WAT lipolysis is supported by the finding that deletion of the murine insulin receptors in both the brain and periphery results in severe lipodystrophy, yet deletion of insulin receptors in peripheral tissues (including WAT) alone but not the brain leads to only mild changes in adiposity (Koch et al., 2008). Therefore, we hypothesized that insulin acts in the brain to suppress lipolysis and induce lipogenesis, maintaining adipose functionality.

RESULTS

To test whether brain, and specifically hypothalamic insulin signaling, regulates WAT lipolysis and lipogenesis, we raised insulin levels locally in the brain by infusing insulin directly into the third ventricle (intracerebroventricularly, or i.c.v.) or the mediobasal hypothalamus (MBH) of male Sprague-Dawley (SD) rats via stereotactic cannulae. To first delineate insulin signaling in specific hypothalamic nuclei within the MBH, we performed acute insulin signaling studies in i.c.v. and MBH insulin-infused

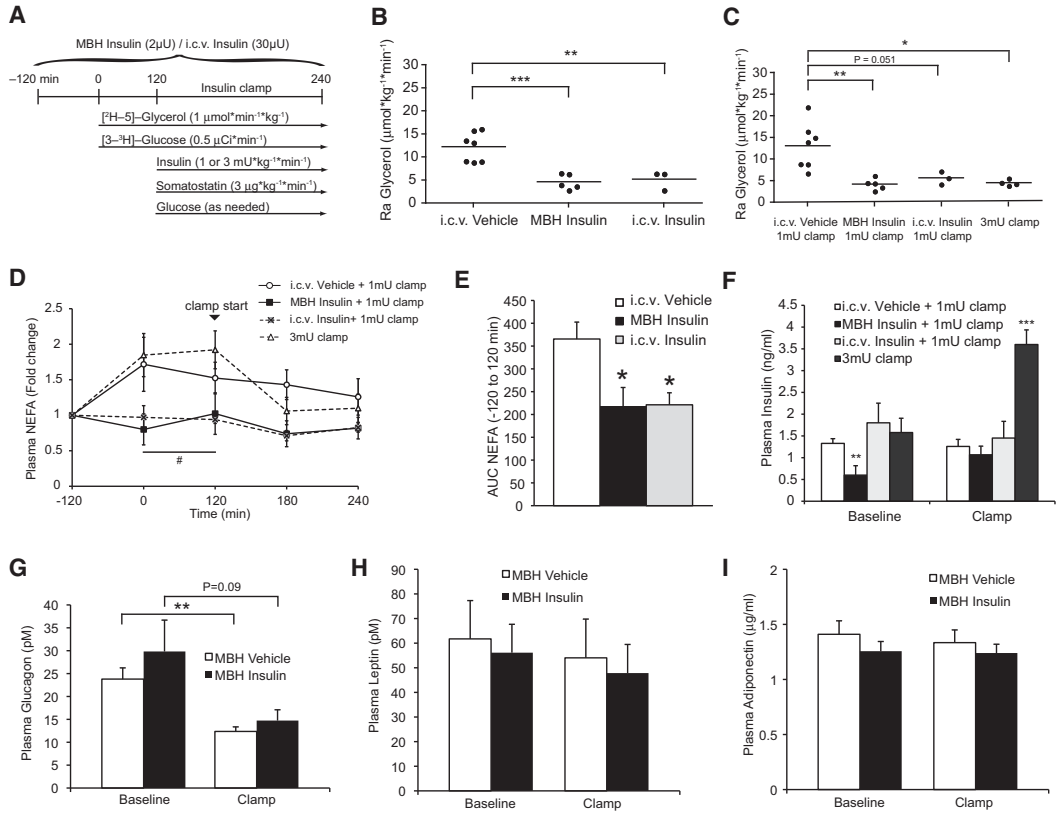

Figure 1. Brain Insulin Suppresses Whole-Body Lipolysis

(A) Experimental protocol of the euglycemic clamp studies of SD rats. i.c.v. or MBH insulin infusions were performed during basal insulin clamps (1 mU · kg^{-1} · min^{-1}) and compared to rats subjected to hyperinsulinemic clamps (3 mU · kg^{-1} · min^{-1}), while glycerol and glucose fluxes were determined through tracer dilution techniques.

(B and C) Ra glycerol during basal (B) and clamped (C) conditions (n ≥ 3 per group).

(D) Change of plasma NEFA levels compared to baseline during the 6 hr infusion protocol. Arrowhead marks the start of the clamp at time point 120 min (n ≥ 4 per group).

(E) AUC of Figure 1D comparing vehicle to i.c.v. and MBH insulin-infused rats prior to the start of the clamp study (time point from −120 to 120 min, n ≥ 4 per group).

(F) Plasma insulin levels during baseline (time point from −120 to 120 min preclamp period) and the clamp (from 120 to 240 min, n ≥ 6 per group).

(G–I) Plasma glucagon, leptin, and adiponectin levels of MBH vehicle- and insulin-infused rats at baseline and the clamp (n ≥ 6). All error bars are SEM; *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle + 1 mU · kg^{-1} · min^{-1} clamp group if not otherwise indicated. #p < 0.05 versus vehicle + 3 mU · kg^{-1} · min^{-1} clamp group (see also Figure S1).

rats and analyzed punch biopsies of the arcuate nucleus (ARC) and the ventromedial hypothalamus (VMH) by western blot at doses that have been commonly used by others (Carvalheira et al., 2003; Rahmouni et al., 2004; Roman et al., 2005). Brain insulin infusion increased insulin receptor phosphorylation and activated downstream targets such as Akt, glycogen synthase kinase 3 (Gsk3), and extracellular signal-regulated kinase (ERK) 1/2 predominantly in the ARC compared to the VMH (Figures S1A and S1B). Next, we infused insulin i.c.v. or intraparenchymally into the MBH for a 6 hr time period to study the role of brain insulin in the regulation of lipolysis in vivo. To reduce the likelihood of pharmacological effects of the insulin doses administered, we chose a dose of insulin that is more than 15,000-fold lower than those commonly used for i.c.v. insulin infusions (Air et al., 2002; Brief and Davis, 1984; Rahmouni et al., 2004). Since the intracerebral infusion of hormones can alter pancreatic insulin secretion and circulating glucose levels, we subjected the animals to pancreatic clamps (protocol depicted in Figure 1A) to maintain euglycemia during the brain infusion of insulin or artificial cerebrospinal fluid (vehicle) (Figure S1C). To compare the effects of isolated brain insulin signaling with the effects of systemic insulin (which includes the direct effects of insulin on adipocytes), we either kept plasma insulin at baseline levels (1 mU · kg^{-1} · min^{-1}) or induced hyperinsulinemia (3 mU · kg^{-1} · min^{-1}) to mimic the fed state (Figure 1F). As glucose metabolism and lipolysis are regulated by insulin, both were assessed simultaneously by employing tracer dilution techniques to determine glucose and glycerol fluxes utilizing [3-^{3}H]glucose and [^{2}H-5]glycerol, respectively.

Brain Insulin Suppresses Whole-Body Lipolysis

Both i.c.v. and MBH insulin administration markedly suppressed the rate of appearance (Ra) of glycerol under basal and clamped conditions, indicating that brain insulin, and more specifically

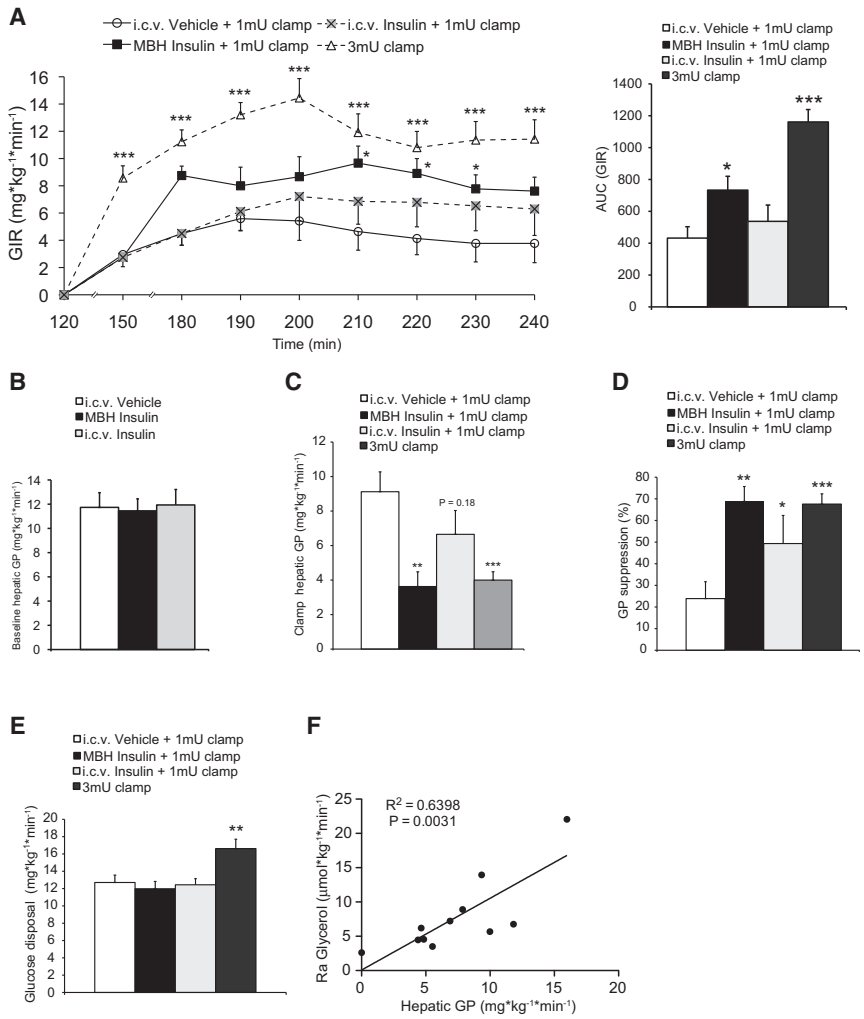

Figure 2. MBH Insulin Suppresses Hepatic GP, which Correlates with Lipolytic Flux

(A) GIR required to maintain euglycemia. Right, AUC of line graph on the left (n ≥ 6 per group).

(B) Baseline hepatic GP (n ≥ 6 per group).

(C) Clamp hepatic GP (n ≥ 6 per group).

(D) Percent suppression of GP during the clamp from baseline (n ≥ 6 per group).

(E) Rate of glucose disposal during the clamp (n ≥ 6 per group).

(F) Correlation between Ra glycerol and hepatic GP during the 1 mU clamp (n = 11). All error bars are SEM; *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle + 1 mU · kg^{-1} · min^{-1} clamp group (see also Figure S2).

MBH insulin signaling, suppresses lipolysis (Figures 1B and 1C). Systemic glucose and insulin levels were not different between i.c.v. vehicle-, i.c.v. insulin-, and MBH insulin-infused rats during a 1 mU · kg^{-1} · min^{-1} basal pancreatic clamp, while insulin levels were raised about 3-fold in the 3 mU · kg^{-1} · min^{-1} hyperinsulinemic clamp group (Figures S1C and S1F). Thus, brain insulin infusion inhibited lipolysis independently of increases in peripheral insulin levels. Hyperinsulinemia induced by a 3 mU · kg^{-1} · min^{-1} clamp decreased the Ra glycerol by about 65% compared to a 1 mU · kg^{-1} · min^{-1} clamp in vehicle-infused animals (Figure 1C). Thus, at the doses administered, brain insulin infusion inhibited lipolysis to an extent similar to that achieved with peripheral hyperinsulinemia. In a separate series of studies, we repeated MBH insulin infusions and compared this to MBH vehicle-infused animals. Again, MBH insulin markedly suppressed Ra glycerol compared to MBH vehicle-infused rats independently of circulating glucose and insulin levels (Figures S2A, S2D, and S2E).

These dynamic changes in the rates of whole-body lipolysis were reflected by changes in static measurements of plasma parameters of fatty acid metabolism, albeit to a lesser degree. NEFA levels trended to increase during the basal clamp period in vehicle-infused animals, which is due to the transitioning of the animal into the fasting state and the flushing of catheters with saline and heparin, a lipoprotein lipase activator, commonly used to prevent clotting of the vascular access. This rise in plasma NEFA levels was prevented by both MBH and i.c.v. insulin administration (Figure 1D), which was also reflected in a significantly lower AUC (Figure 1E). Of note, the effects of centrally administered insulin closely mimicked the effects of systemic insulin infusion in humans, where the heparin-induced increase in plasma NEFAs is completely blunted by insulin treatment (Chaudhuri et al., 2007). Plasma glycerol and triglyceride levels trended in the same direction, although this failed to reach statistical significance (Figures S1D and S1E). As expected, due to the administration of somatostatin, glucagon levels decreased during the clamp, but neither glucagon nor the levels of the adipokines leptin and adiponectin were different between groups (Figures 1G–1I).

Lipolytic Flux Tightly Correlates with Hepatic Glucose Production

Lipolytic flux can potentially regulate hepatic glucose production (GP) through two mechanisms (Mittelman and Bergman, 2000; Rebrin et al., 1996): (1) by providing the liver with the gluconeogenic precursor glycerol and (2) by enabling the synthesis of high-energy NADH substrates required during gluconeogenesis through β-oxidation of NEFAs (Hers and Hue, 1983). Therefore, we simultaneously assessed glucose fluxes in the same studies to uncover a potential interdependence. MBH insulin-infused rats required higher glucose infusion rates (GIRs) compared to vehicle-infused rats during the clamp (Figures 2A and S2F) and suppressed hepatic GP to an extent similar to rats treated with systemic hyperinsulinemia (Figures 2C, 2D, and S2B), which is consistent with prior reports (Pocai et al., 2005). Notably, despite the ability of MBH insulin to suppress hepatic GP during the clamp period, circulating glucose levels (Figures S1C and S2D)

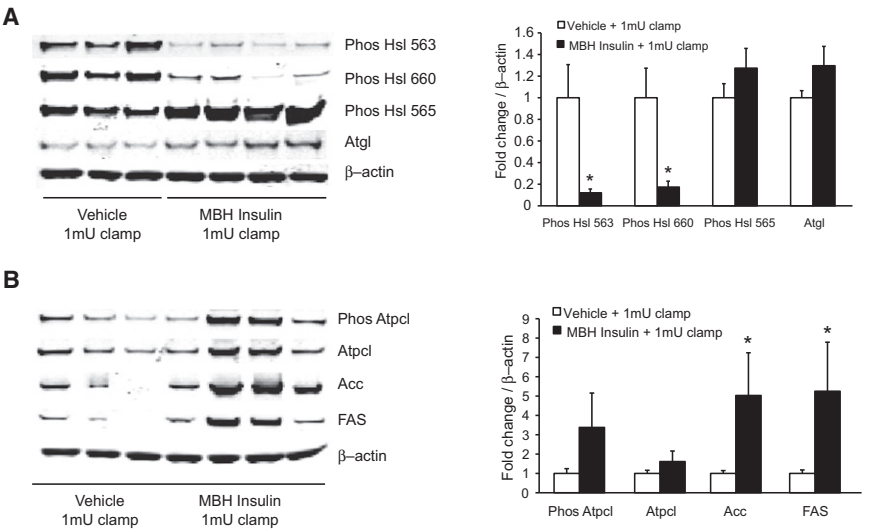

Figure 3. MBH Insulin Suppresses Hsl Activation and Increases Lipogenic Protein Expression

(A and B) MBH insulin suppresses lipolysis and induces lipogenesis in WAT. Left, representative western blot analyses of epididymal fat pads from clamped animals. Right, quantification of the western blot analyses ($n \geq 5$ per group).

(C) Lipid-droplet-depleted cytosolic triglyceride hydrolase activity ($n \geq 8$ per group; one-tailed t test was applied).

(D) FAS activity measured in perirenal fat depots ($n = 4$ per group; one-tailed t test was applied). All error bars are SEM; *$p < 0.05$ vehicle group (see also Figure S3).

and hepatic GP (Figure 2B) did not change during the basal period where MBH insulin was infused, but not IV somatostatin. Glucose disposal into peripheral tissues, as assessed through the use of [3-^{3}H]glucose tracer dilution technique, was not altered by central insulin infusion during basal clamps (Figures 2E and S2C). As expected, systemic hyperinsulinemia increased glucose utilization in peripheral tissues like muscle and WAT, which is mainly a function of the direct effects of insulin on both tissues (Figure 2E). Of note, the Ra glycerol tightly correlated with hepatic GP during the 1 mU clamps (Figure 2F), while neither of these parameters correlated with circulating insulin levels (Figures S2H and S2I). Since we controlled glucose levels through euglycemic clamps, we can rule out that hepatic GP drives WAT lipolysis in these studies. However, the tight correlation between lipolytic flux and hepatic GP during the MBH insulin infusion as well as the fact that MBH insulin did not change hepatic gluconeogenic gene expression of *Pck1* and *G6pc* (Figure S2G) suggests that one of the mechanisms through which hypothalamic insulin regulates hepatic GP is by controlling lipolytic flux from WAT.

MBH Insulin Decreases Systemic Lipolysis by Decreasing Hsl and Atgl Activation in WAT by Suppressing Sympathetic Outflow

To delineate the molecular mechanisms through which hypothalamic insulin regulates lipolysis, we analyzed the expression and activation state of lipolytic proteins such as hormone-sensitive lipase (Hsl, encoded by *Lipe*). Hsl hydrolyzes diacylglycerols to monoacylglycerols and serves as a marker for SNS outflow to WAT (Bartness et al., 2010; Buettner et al., 2008). Consistent with the glycerol flux data, we found that central insulin suppressed the activation state of Hsl (Figure 3A). Protein kinase A (PKA) activates Hsl through phosphorylation of Ser563 and Ser660 (Anthonsen et al., 1998). Central insulin decreased phosphorylation at both these sites, while phosphorylation of Ser565, which inactivates Hsl (Anthonsen et al., 1998), trended to be increased (Figure 3A). Hsl is phosphorylated at Ser565 by AMP-activated protein kinase (Ampk) (Anthonsen et al., 1998; Garton and Yeaman, 1990), which has been shown to inhibit adipocyte lipolysis (Anthony et al., 2009). Indeed, phospho-Ampkα (Thr172) levels, an indicator of Ampk activation, were increased in WAT from MBH insulin-infused animals (Figure S3A). Next, we assessed triglyceride hydrolase activity in lipid-droplet-depleted adipose tissue homogenates harvested from the same MBH insulin- and vehicle-infused rats. The activation of Hsl by PKA leads to its translocation from the cytosol to the surface of the lipid droplet (Egan et al., 1992; Granneman et al., 2007). As total Hsl levels in total cell extracts were not different between MBH insulin- and vehicle-infused rats (data not shown), activation of Hsl should lead to its cytosolic depletion. Since MBH insulin infusion suppressed Hsl phosphorylation, we predicted higher cytosolic Hsl activity compared to vehicle-infused controls after lipid droplet removal, which indeed was the case (Figure 3C). Thus, MBH insulin suppresses Hsl activity in WAT by inhibiting its phosphorylation and thereby likely its translocation to lipid droplets.

Adipose tissue triglyceride lipase (Atgl, encoded by *Pnpla2*) hydrolyzes triacylglycerols to diacylglycerols and, together with Hsl, accounts for over 90% of the WAT acyl-hydrolase activity (Zimmermann et al., 2004). MBH insulin infusion did not alter Atgl protein expression in WAT (Figure 3A). However, Atgl activity is not solely determined by total protein levels, but is also regulated through cAMP-dependent and -independent posttranslational modification. Atgl activity is increased upon β-adrenergic stimulation via a posttranslational mechanism

(Haemmerle et al., 2006). This activation critically depends on the phosphorylation of the lipid-droplet-associated protein perilipin, which involves the dissociation of α/β hydrolase domain containing protein 5 (ABHD5; also known as *comparative gene identification-58* [CGI-58]) from perilipin and its subsequent activation of Atgl (Granneman et al., 2007; Miyoshi et al., 2007; Zimmermann et al., 2009). Phospho-perilipin, as assessed by a PKA substrate motif antibody, trended ($p = 0.053$) to be suppressed in WAT after MBH insulin infusion (Figure S3B). These results suggest that in addition to suppressing Hsl activity, MBH insulin reduces Atgl activation by decreasing perilipin phosphorylation and thereby favoring CGI-58 retention. Thus, brain insulin regulates Hsl activity and likely Atgl activation via PKA.

Insulin antagonizes cAMP signaling through activation of phosphodiesterase 3B (PDE3B) in an Akt-dependent manner (Degerman et al., 2003). It has been shown that hypothalamic leptin signaling can increase insulin signal transduction as assessed by Akt phosphorylation in peripheral organs like the liver (German et al., 2009). Both leptin and insulin regulate autonomic outflow within the MBH (Buettner and Camacho, 2008), which could influence insulin signaling—through crosstalk from cAMP signaling, for example. Although we controlled circulating insulin levels during the clamp studies, it is still conceivable that hypothalamic insulin could enhance insulin signaling in WAT. Thus, we tested whether MBH insulin alters Akt activation in WAT as assessed by Akt phosphorylation at Ser473. We found that MBH insulin did not change Akt phosphorylation in WAT (Figure S3C), indicating that the effects of brain insulin on WAT metabolism occur independently of changes in peripheral insulin signaling.

Brain Insulin Induces Lipogenesis in WAT

Lipogenesis in WAT has recently been shown to regulate whole-body insulin sensitivity by generating palmitoleate, which improves insulin sensitivity (Cao et al., 2008). Furthermore, we have found that lipogenesis in WAT is upregulated in metabolically beneficial states, even though adiposity is decreased. For example, lipogenesis is elevated in young versus aged rats (Figure S3D), calorically restricted (CR) aged rats versus ad libitum (AL) fed aged rats (Figure S3D), and exercised versus sedentary mice (data not shown). Since hyperinsulinemia induces lipogenesis in WAT (Assimacopoulos-Jeannet et al., 1995), we asked if brain insulin signaling is sufficient to increase lipogenesis in WAT in the absence of peripheral hyperinsulinemia and assessed lipogenic protein expression in the same tissue lysates that had been used for the Hsl western blot analysis (Figure 3A). MBH insulin increased the expression of the key lipogenic proteins fatty acid synthase (FAS, encoded by *Fasn*) and acetyl-CoA carboxylase (Acc, encoded by *Acaca*) (Figure 3B). Adipose tissue FAS enzyme activity of MBH insulin-infused rats was increased by 60%, just below statistical significance ($p = 0.086$) (Figure 3D). Protein expression of ATP-citrate lyase (Atpcl) and its phosphorylation, a marker of the activated state, trended to be increased (Figure 3B). These findings implicate that, in vivo, (1) WAT lipogenesis and lipolysis are inversely regulated by hypothalamic insulin, and (2) MBH insulin seems to oppose the acute actions of MBH leptin. We have shown in a prior study that MBH leptin induces lipolysis and inhibits lipogenesis by increasing sympathetic outflow to WAT (Buettner et al., 2008).

MBH Insulin Dampens SNS Activity

Therefore, to delineate whether the effects of MBH insulin on WAT metabolism are mediated via the autonomic nervous system, we performed surgical denervation as well as pharmacological sympathectomy of rat epigonadal fat pads using 6-hydroxydopamine (6-OHDA). Both surgical denervation and pharmacological sympathectomy suppressed basal WAT Hsl activation, a marker for sympathetic outflow to WAT (Bartness et al., 2010; Buettner et al., 2008). This effect was replicated with MBH insulin infusion (Figure 4A). Although this experiment is not an ultimate proof of a linear pathway, these data suggest that hypothalamic insulin regulates Hsl activation via suppression of SNS outflow to WAT.

The Acute Inhibition of Brain Insulin Signaling Is Sufficient to Unrestrain Lipolysis

We next asked if the acute induction of brain insulin resistance is sufficient to alter lipolysis. S961 is a recently identified peptide inhibitor that blocks insulin binding to the insulin receptor, while not inhibiting insulin or insulin-like growth factor (IGF)-1 binding to the IGF receptor or IGF-1 binding to the insulin receptor (Schäffer et al., 2008). First, we validated that S961 blocked insulin-induced insulin receptor phosphorylation in the MBH after i.c.v. infusion (Figures 4B), which also obliterated downstream signaling (Figure S4A). To assess if the inhibition of endogenous insulin in the MBH is sufficient to unrestrain lipolysis, we infused either the insulin receptor antagonist S961 or vehicle into male SD rats for 4 hr, while simultaneously infusing [^{2}H-5]glycerol tracer systemically (Figure 4C). During the infusion study, systemic glucose and insulin levels were similar between groups (Figures S4B and S4C). Indeed, inhibiting endogenous insulin signaling in the MBH doubled the Ra glycerol (Figure 4D), suggesting that hypothalamic insulin resistance can contribute to the unrestrained lipolysis seen in the insulin-resistant state. Since increased GP is a major component of widespread SNS activity (Gellhorn, 1954) and glucose levels throughout the entire infusion experiment were not different in S961 versus vehicle-infused rats, it is unlikely that S961 is an unspecific activator of the SNS.

The denervation studies shown in Figure 4A suggested that MBH insulin reduces WAT lipolysis via the autonomic nervous system, since MBH insulin suppressed Hsl phosphorylation, comparable to chemical sympathectomy. Here, we further tested this concept by asking if the unrestrained lipolysis induced by the acute inhibition of insulin signaling in the MBH depended on intact sympathetic innervations. To this end, we induced systemic sympathectomy in male SD rats by injecting 6-OHDA. After a short recovery period, we subjected the animals to the same infusion studies depicted in Figure 4C in order to test if MBH S961 would still be able to increase Ra glycerol. As expected, systemic sympathectomy obliterated the prolipolytic effects of the insulin antagonist (Figure 4E). Importantly, WAT norepinephrine (NE) content was markedly reduced, verifying the sympathectomy (Figure 4F). Ra glycerol trended to be lower in the denervated animals compared to sham controls, suggesting that sympathectomy lowers prolipolytic input to WAT. Thus, these results provide further support for the concept that MBH insulin signaling controls WAT lipolysis by dampening SNS activity.

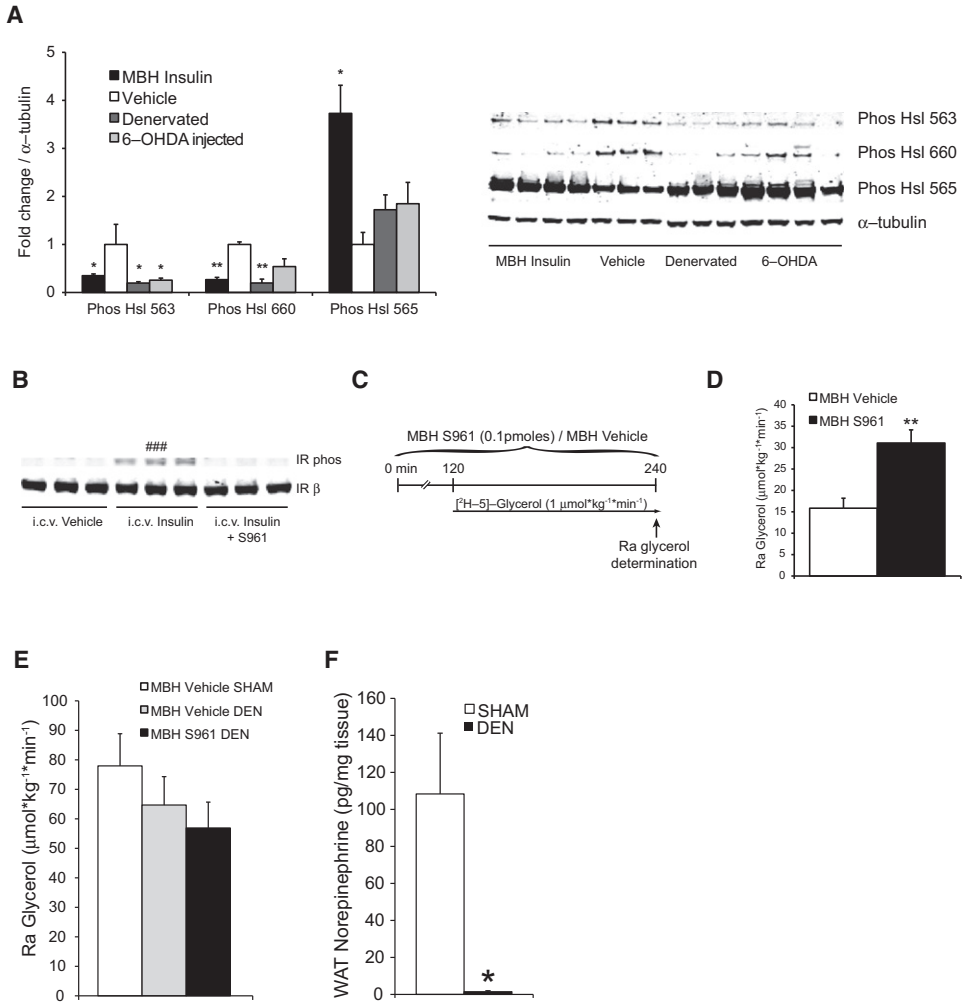

Figure 4. Acute Inhibition of Endogenous MBH Insulin Signaling Is Sufficient to Unrestrain Lipolysis in WAT, which Depends on Sympathetic Innervations

(A) MBH insulin suppresses Hsl activation to a degree similar to surgical denervation or selective pharmacological sympathectomy of the epididymal fat pad, indicating that insulin suppresses lipolysis through a reduction of sympathetic nervous system outflow to WAT. Left, quantifications; right, western blot analyses ($n \geq 3$ per group).

(B) Validation of the insulin receptor antagonist in vivo. S961 was coinfused with insulin in equimolar amounts into the i.c.v. of male SD rats. S961 blocked insulin-induced MBH insulin receptor phosphorylation (n = 3 per group).

(C) Schematic study outline. Male SD rats were infused with glycerol tracer and received either vehicle or S961 for 4 hr into the MBH.

(D) Ra glycerol of MBH vehicle- and S961-infused rats as per protocol depicted in Figure 4C ($n \geq 4$ per group).

(E) Ra glycerol of sham and pharmacologically sympathectomized rats that were infused with either MBH S961 or vehicle. A protocol similar to that depicted in (C) was used ($n \geq 6$ per group).

(F) WAT NE levels in sham versus 6-OHDA denervated rats (n = 4 per group). All error bars are SEM; *$p < 0.05$, **$p < 0.01$ versus vehicle group. ###$p < 0.001$ versus i.c.v. vehicle and i.c.v. insulin + S961 group (see also Figure S4).

Genetic Deletion of the Neuronal Insulin Receptor Increases Lipolysis in Mice and Impairs the Metabolic Switch from Fasting to Refeeding

To test if neuronal insulin signaling mediates the role of brain insulin in regulating lipolysis, we studied glycerol fluxes in neuronal insulin receptor knockout (NIRKO) and littermate lox-lox control mice (Figure S5A) during hyperinsulinemic-euglycemic clamp studies (Figures S5B and S5C, protocol depicted in Figure 5A). NIRKO mice have been reported to be susceptible to diet-induced obesity, although their body weight (BW) is not altered when they are young and fed a standard chow diet. Furthermore, they develop mild insulin resistance when they get older than 6 months (Brüning et al., 2000). We find that 4-month-old NIRKO mice required equal GIR during a hyperinsulinemic clamp, indicating no or only mild impairment of glucose homeostasis in young NIRKO mice (Figure S5D). In the fasted state, NIRKO mice exhibited a 2-fold higher Ra glycerol compared to lox-lox littermate controls (Figure 5B), although insulin levels were not different (Table S1), demonstrating that neuronal insulin signaling controls basal lipolytic rate. Induction

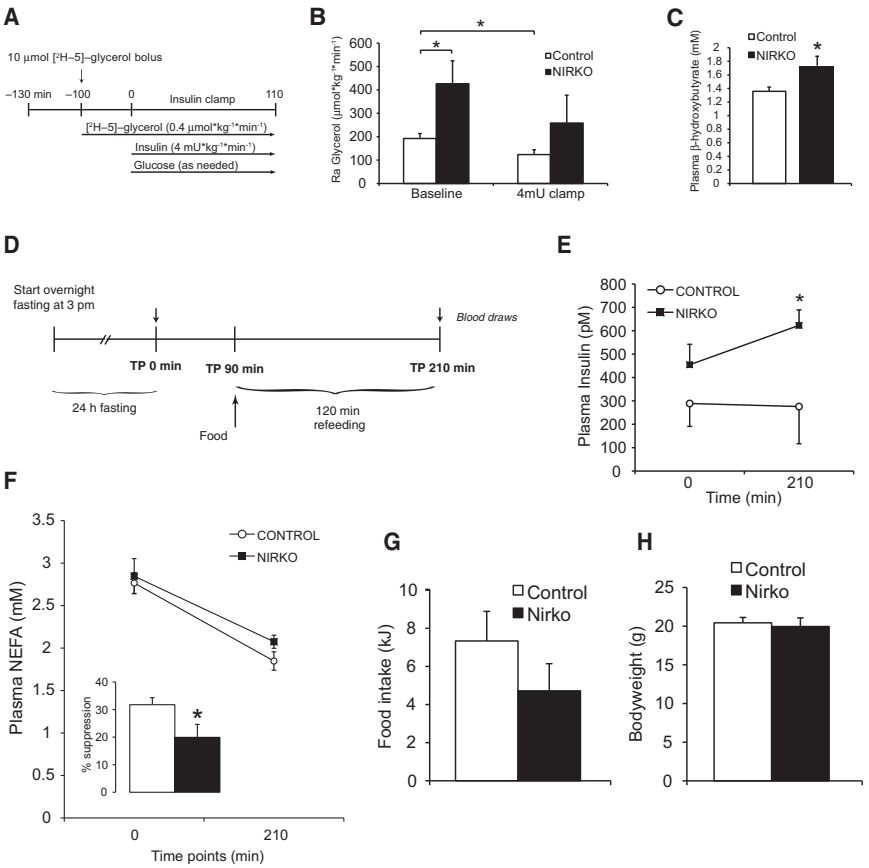

Figure 5. Genetic Disruption of Neuronal Insulin Signaling Increases Whole-Body Lipolytic Flux and Impairs the Switch from Fasting to Refeeding

(A) Schematic representation of the clamp studies in NIRKO mice. Following a 16 hr fast, NIRKO and littermate control mice were subjected to a 110 min 4 mU · kg^{-1} · min^{-1} hyperinsulinemic-euglycemic clamp study.

(B) Baseline and clamp glycerol fluxes as assessed by [^{2}H-5]glycerol tracer infusion are increased in the NIRKO mice ($n \geq 5$ per group).

(C) Plasma β-hydroxybutyrate levels are elevated in the NIRKO mice following a 16 hr fasting challenge ($n = 9$ per group).

(D) Depiction of the fasting-refeeding protocol.

(E) Plasma insulin before and after refeeding ($n \geq 8$ per group).

(F) Plasma NEFA levels before and after refeeding. Insert depicts percent suppression of plasma NEFA levels after food intake ($n \geq 7$ per group).

(G) Food intake during refeeding ($n \geq 9$ per group).

(H) Bodyweights after fasting ($n \geq 9$ per group). All error bars are SEM; *$p < 0.05$ versus littermate control mice (see also Figure S5 and Table S1).

of hyperinsulinemia during a 4 mU · kg^{-1} · min^{-1} euglycemic clamp raised insulin levels equally in both groups (Figure S5B), and while glycerol fluxes were suppressed in both NIRKO and control mice (presumably through the direct actions of insulin on adipocytes), the Ra glycerol remained higher in the NIRKO mice (Figure 5B) while glucose and glucagon levels were not different between groups (Figures S5C and Table S1). These data indicate that lipolytic flux is exquisitely sensitive to brain insulin action, more so than hepatic glucose fluxes. Changes in levels of plasma NEFA and glycerol did not show significant differences (Figure S5E). Since unrestrained lipolysis is expected to increase ketogenic substrate flux to the liver, we further assessed whether NIRKO mice display increased ketogenesis. Indeed, NIRKO mice challenged with a 16 hr fast had elevated β-hydroxybutyrate levels compared to control animals (Figure 5C), supporting the concept that brain insulin signaling can regulate both lipolysis and ketogenesis. Plasma NEFA and glycerol levels were not different between groups following the 16 hr fast (Figures S5F and S5G). The transition from fasting to refeeding is a physiological challenge that requires adipose tissue to readily switch from NEFA release to NEFA retention. To assess the role of neuronal insulin receptor signaling in this metabolic adaptation, we fasted NIRKO and control littermates overnight and then refed them during the onset of their feeding cycle (protocol depicted in Figure 5D). This allowed us to assess the suppression of NEFA release after refeeding. While fasting NEFAs were not different between NIRKO and control mice, the suppression of NEFAs following a 2 hr refeeding period in NIRKO mice was significantly lower (Figure 5F), despite higher peripheral insulin levels after feeding (Figure 5E). Insulin levels of control mice were unchanged after feeding, which is consistent with prior reports that showed that lean insulin-sensitive C57BL/6 mice do not exhibit a change in insulin levels after oral glucose administration (Andrikopoulos et al., 2008). Bodyweights and food intake during refeeding were not different between groups (Figures 5G and 5H). These data indicate that the neuronal insulin receptor regulates lipolysis in WAT during the fasting-to-feeding transition.

Neuronal Insulin Receptor Signaling Regulates De Novo Lipogenesis in WAT

Consistent with our findings from the rat studies where MBH insulin increased lipogenic protein expression, loss of brain insulin signaling in the NIRKO mice decreased Acc and FAS protein expression as well as activated phospho-Atpcl in WAT under clamped and overnight-fasted conditions (Figures 6A and S5H), suggesting that the lifelong absence of brain insulin signaling disrupts WAT lipogenesis. To test whether the decreased WAT lipogenic enzyme expression in the NIRKO mice translated into alterations in WAT fatty acid composition, we generated fatty acid lipid profiles from epididymal fat pads of fasted control and NIRKO mice. We found a marked suppression of several fatty acid species (14:0, 14:1n5, 16:0, 16:1n7) associated with de novo lipogenesis in the epididymal fat depots of the NIRKO mice (Figure 6C), as well as a lower de novo lipogenesis index (Figure 6B), which is used as a marker for lipogenesis (Chong et al., 2008; Hudgins et al., 1996). However, oleate (18:1n9) and linoleic acid (18:2n6), two of the most

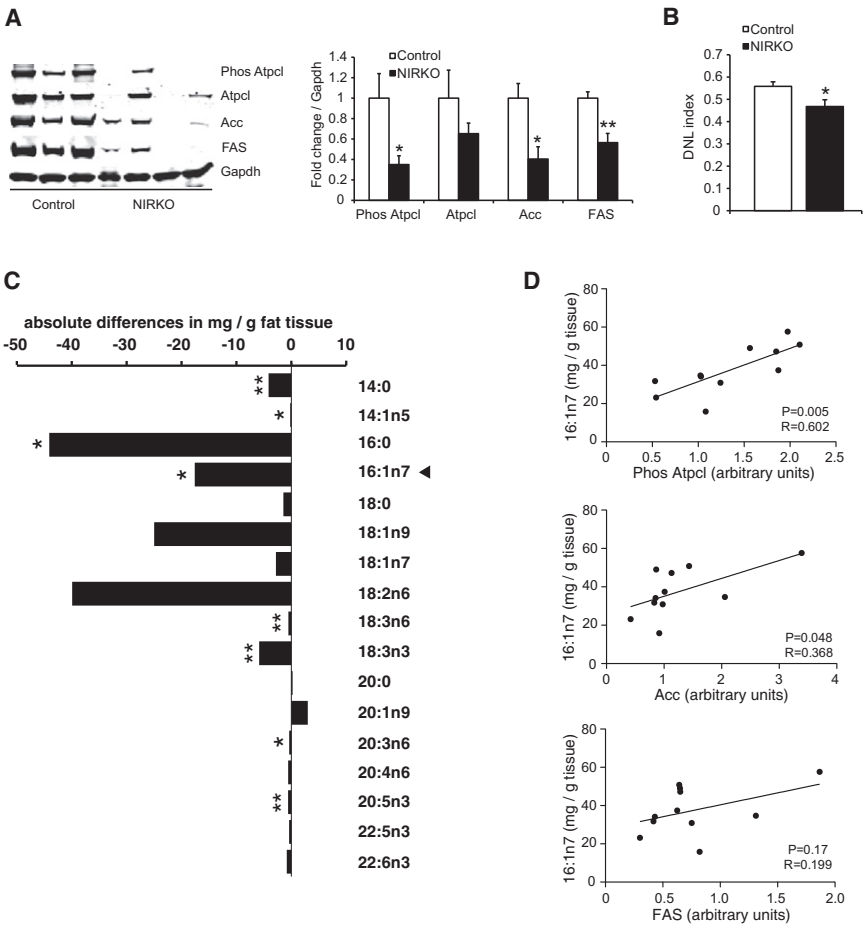

Figure 6. Loss of Neuronal Insulin Receptor Signaling Impairs De Novo Lipogenesis in WAT

(A) Left, representative western blot analyses of lipogenic protein expression and the activation state of Atpcl in epididymal fat pads obtained at the end of the clamp study. Right, quantification of western blot data compared to littermate control mice ($n \geq 5$ per group).

(B) De novo lipogenesis (DNL) index calculated using the ratio of palmitic (16:0) to linoleic acid (18:2n6) ($n \geq 5$ per group).

(C) Differences of WAT fatty acid species of overnight-fasted NIRKO and control mice. Arrowhead marks palmitoleate (n = 6 per group).

(D) Correlation between the expression of lipogenic proteins and palmitoleate levels (n = 11). All error bars are SEM; *$p < 0.05$, **$p < 0.01$ versus control mice.

abundant fatty acids in WAT, were not significantly changed, suggesting that triglyceride content was not different between groups. Notably, the insulin-sensitizing lipokine palmitoleate (16:1n7), which is produced during de novo lipogenesis in WAT (Cao et al., 2008), was decreased in the NIRKO mice (Figure 6C). Furthermore, palmitoleate levels in WAT correlated closely with the expression of the lipogenic enzyme Acc and the activation state of Atpcl in the fasted state, further supporting the notion that brain insulin regulates palmitoleate synthesis through regulation of WAT lipogenesis (Figure 6D). These findings assign a critical role to neuronal insulin signaling in maintaining WAT function. We speculate that hypothalamic insulin resistance could contribute to the decreased lipogenic protein expression in WAT observed in obesity. This in turn leads to decreased production of the insulin-sensitizing fatty acid palmitoleate, further worsening systemic insulin resistance.

DISCUSSION

WAT has emerged as a critically important organ for whole-body glucose and lipid homeostasis. WAT is an important metabolic sink, clearing and storing circulating lipids, thereby protecting other organs from ectopic lipid accumulation. In addition, WAT is an important source of adipokines like leptin and adiponectin, and inflammatory mediators like TNF-α and IL-6 that circulate and can impair insulin signaling in distant organs including the hypothalamus (Zhang et al., 2008). During fasting, WAT is the main source of NEFAs that provide energy substrates to muscle and liver. Unrestrained lipolysis during the absorptive state induces lipotoxicity and a low-grade inflammation that is commonly associated with obesity and diabetes (Boden, 2006). Lastly, de novo lipogenesis in WAT produces insulin-sensitizing fatty acid species like palmitoleate (Cao et al., 2008).

Our studies establish that neuronal and in particular hypothalamic insulin action is a critical regulator of WAT metabolism. We demonstrate that brain insulin action restrains lipolysis by reducing sympathetic outflow to WAT and controls de novo lipogenesis in adipose tissue. Impaired brain and hypothalamic insulin signaling increases lipolytic flux and decreases de novo lipogenesis, hampering the production of the insulin-sensitizing fatty acid palmitoleate. We come to this conclusion after delineating the role of brain insulin in regulating WAT metabolism in two independent models: (1) SD rats, where we either increased or inhibited brain or MBH insulin signaling, and (2) mice with a lifelong disruption of neuronal insulin signaling. The molecular mechanism through which MBH insulin regulates WAT metabolism within the adipocyte is comprised of an increase in lipogenic protein expression and activity and a decrease in the activation state of lipolytic enzymes like Hsl (see proposed model in Figure 7). Whether other antilipolytic regulators such as adenosine, prostaglandin E_2, neuropeptide Y, or lactate participate in the regulation of WAT metabolism by brain insulin remains to be determined (Ahmed et al., 2010; Jaworski et al., 2009; Lafontan and Langin, 2009).

These findings that ascribe a role of brain insulin signaling in the regulation of WAT functionality not only have implications for lipid metabolism, but are also likely to be relevant in glucose homeostasis, since lipolytic flux can contribute to hepatic gluconeogenesis (Mittelman and Bergman, 2000; Rebrin et al., 1996).

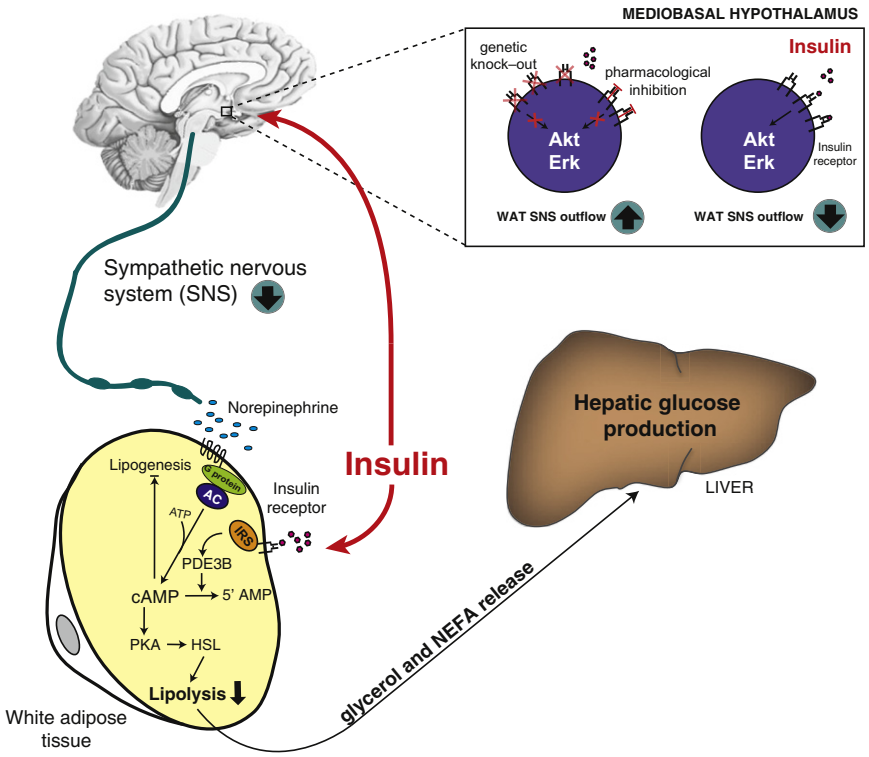

Figure 7. Proposed Model of the Role of Brain Insulin in Regulating WAT Metabolism

Insulin inhibits WAT lipolysis through both direct and indirect effects: insulin binding to the insulin receptor expressed on adipocytes results in inactivation of PDE3B, leading to the degradation of cAMP (Degerman et al., 1990; Smith et al., 1991). We propose that in addition to the direct effects of insulin on adipocytes, hypothalamic insulin signaling suppresses lipolysis and induces lipogenesis indirectly by dampening SNS outflow to WAT. The reduction in lipolysis contributes to a decrease in hepatic GP by limiting the flux of the gluconeogenic precursor glycerol and NEFAs, which provide energy substrates for gluconeogenesis.

Indeed, our data indicate that lipolytic flux is exquisitely sensitive to brain insulin action, more so than hepatic glucose fluxes. MBH insulin infusion in rats did not alter hepatic GP even after 4 hr, while Ra glycerol flux was already altered. Only after somatostatin infusions were started did an effect on hepatic GP become apparent.

There is growing evidence that in obesity and diabetes, lipogenic capacity of adipose tissue is reduced in rodents as well as humans (Diraison et al., 2002; Moraes et al., 2003; Nadler et al., 2000). The stimulatory effects of brain insulin on WAT lipogenesis, plus the finding that the absence of the neuronal insulin receptor impairs the lipogenic capacity of WAT, suggest that the dysregulation of WAT adipose tissue lipogenesis in obesity is at least in part a function of brain insulin resistance.

Furthermore, our findings suggest that brain insulin action has important anabolic function in WAT maintenance, which is independent of, but complements, cell-autonomous effects of insulin on adipocytes. In further support of this concept, a recent study showed that the presence of the brain insulin receptor is critically important to prevent lipodystrophy in mice (Koch et al., 2008). The same study showed that chronic i.c.v. insulin infusion in mice leads to increased fat pad mass and hypertrophy of adipocytes, which the authors ascribed to increased lipogenesis. Equally if not more important in the control of adiposity is likely the regulation of WAT lipolysis through brain insulin signaling. We draw this conclusion from the finding that denervation of WAT leads to no change in lipogenic protein expression, but completely abrogates Hsl activation, leading to increased adipose depot mass (Buettner et al., 2008). The absence of WAT renders humans (Hegele, 2003; Petersen et al., 2002) and mice (Shimomura et al., 1999) severely insulin resistant due to the ectopic accumulation of lipids in tissues such as muscle and liver. Ectopic lipids impair insulin signaling and induce a proinflammatory state. In an insulin-sensitive organism, WAT is metabolically flexible and able to readily switch from a fatty acid-storing to a fatty acid-releasing mode according to metabolic needs.

Our studies raise several questions. One is which neuronal subtype within the CNS and the MBH mediates the effects of insulin on the regulation of WAT metabolism. Several neuron-specific insulin receptor knockout models have been generated (Belgardt et al., 2009), although to our knowledge an analysis of lipolytic flux or WAT lipogenesis has not been undertaken as of yet. Judging from the energy balance and glucose homeostasis phenotype of these diverse models, it appears that the effects of brain insulin are not explained by a single neuronal population, but rather through an intricate and currently poorly understood communication within a complex neuronal network where, in some instances, insulin signaling in one neuron type can even antagonize the effects of insulin signaling in another neuronal population (Lin et al., 2010). Thus, even if a specific neuronal KO of the insulin receptor abolishes the ability of brain insulin to regulate WAT metabolism, this may mean that a certain balance within this neuronal network is disturbed, not that this particular neuron population is the chief target and mediator of hypothalamic insulin action. Another question is whether brain insulin signaling or adipocyte insulin signaling is predominant in the regulation of WAT metabolism by systemic insulin. Our studies clearly establish that in the absence of alterations of circulating insulin levels, brain insulin action plays a critical role in the regulation of WAT metabolism.

Furthermore, our findings raise the possibility that hypothalamic insulin action may either (1) regulate both lipolysis and hepatic GP via a suppression of sympathetic outflow to the liver or (2) indirectly regulate hepatic GP by decreasing the flux of glycerol and NEFA to the liver via suppression of lipolysis in WAT.

Finally, conditions in which brain insulin signaling is compromised, such as high-fat feeding (Ono et al., 2008), chronic inflammatory conditions (Zhang et al., 2008), and obesity (Posey et al., 2009; Zhang et al., 2008), are likely to dysregulate the control of

lipolysis and de novo lipogenesis in WAT due to impaired insulin action in the hypothalamus. Thus, hypothalamic insulin resistance unrestrains lipolysis and reduces the lipogenic capacity in WAT, which in turn induces lipotoxicity, resulting in peripheral insulin resistance and thereby perpetuating a vicious cycle.

EXPERIMENTAL PROCEDURES

Animals

Rat experiments were performed in standard-chow-fed (Rodent Diet 5001, LabDiet, St. Louis) male SD rats (Charles River Breeding Laboratories, Wilmington, MA) housed in a temperature- and light-controlled facility in separate cages. Prior to the clamp studies, rats were stereotactically fit with indwelling cannulae targeting the third ventricle (i.c.v.) or the MBH (see Supplemental Experimental Procedures). After a 1 week recovery period, carotid and jugular catheters were implanted for blood sampling and infusion, respectively. Rats were allowed to recover for an additional 4 days and required to return to within 10% of their presurgical BW.

NIRKO mice were generated as described elsewhere (Brüning et al., 2000; Fisher et al., 2005), housed on a 12 hr light-dark cycle, and fed a standard rodent diet (Mouse Diet 9F, PMI Nutrition International, St. Louis). All animal protocols were approved by the IACUC of Mount Sinai School of Medicine.

Rat Pancreatic Clamp Studies

Rat clamp experiments were performed in 10-week-old, conscious, nonrestrained, and ad libitum-fed male SD rats. An i.c.v. (5 μl/hr) or MBH (0.18 μl/hr per side) infusion with either vehicle (artificial cerebrospinal fluid [aCSF]; Harvard Apparatus, Holliston, MA) or insulin (i.c.v., 30 μU; MBH, 2 μU; Humulin R, Lilly) was started and maintained for 360 min. [3-^{3}H]glucose and [^{2}H-5]glycerol tracers were infused as depicted in Figure 1A. The insulin clamp was initiated with a primed continuous infusion of insulin (1 mU or 3 mU · kg^{-1} · min^{-1} infusion; Humulin R, Lilly) while also infusing somatostatin (3 μg · kg^{-1} · min^{-1}). We collected plasma samples before and at the end of the clamp to determine Ra glycerol during baseline and clamped period. See Supplemental Experimental Procedures for a more detailed description.

Local Denervation Experiment

Epididymal fat pads were denervated as previously described (Buettner et al., 2008). Rats were infused for 4 hr with either MBH insulin (1.44 μU) or aCSF (vehicle). See Supplemental Experimental Procedures for a more detailed description.

Insulin Antagonist Infusion Experiment

Eight-week-old male SD rats were equipped with MBH cannulae and catheters. Rats were infused for 4 hr with either aCSF (vehicle) or 0.1 pmoles of the insulin receptor antagonist S961 (a gift from Novo Nordisk, Maaloev, Denmark) (Schäffer et al., 2008) into the MBH, while also employing a [^{2}H-5] glycerol tracer infusion.

Systemic Pharmacological Sympathectomy Experiment

Eight-week-old male SD rats were simultaneously implanted with MBH cannulae and jugular catheters during ketamine-xylazine anesthesia. After a 3 day recovery period, rats were given two IV bolus injections of 6-OHDA or vehicle (0.9% saline containing 0.1% ascorbic acid) 3 days apart (50 mg/kg BW freshly prepared in 0.9% saline containing 0.1% ascorbic acid). Vehicle-injected animals were pair-fed to 6-OHDA-injected rats to match their BWs. Three days following the last IV injection, rats were either infused with MBH vehicle (aCSF) or S961 (0.1 pmoles) using the protocol depicted in Figure 4C. After 2 hr, a [^{2}H-5]glycerol tracer infusion was started and maintained (30 μmol · kg^{-1} · min^{-1} for 4 min bolus followed by 3 μmol · kg^{-1} · min^{-1}) for 2 hr. Blood samples for Ra glycerol analysis were taken at time point (TP) 240 min by tail vein sampling. Only rats recovered within 10% of their presurgical BW were used.

Mouse Clamp Experiments

Mouse clamp studies were performed in 16 hr fasted, 4-month-old, male, conscious, nonrestrained NIRKO and littermate lox-lox control mice that were equipped with femoral artery and right jugular vein catheters. Surgical details are described in Supplemental Experimental Procedures. The protocol consisted of a 100 min tracer equilibration period followed by 110 min clamp period as depicted in Figure 5A. A 10 μmol bolus of [^{2}H-5]glycerol (98 atom percent excess) was given at t = −100 min, followed by a continuous infusion at 4 μmol · kg^{-1} · min^{-1} over 210 min. The insulin clamp was initiated at t = 0 min with a continuous infusion of human insulin (4 mU · kg^{-1} · min^{-1} infusion; Humulin R, Lilly) lasting 110 min. Euglycemia was maintained by measuring glucose every 10 min and infusing 50% dextrose as necessary. Plasma samples were collected before and at the end of the clamp to determine Ra glycerol during baseline and clamped period, respectively. To minimize blood loss, we reinfused spun-down erythrocytes, which were resuspended in saline.

Mouse Fasting-Refeeding Experiments

Ten-week-old NIRKO and littermate control mice were fasted for 24 hr starting at 3 p.m. the day before the experiment, so the refeeding period coincided with the onset of the feeding cycle (protocol depicted in Figure 5D). An initial blood sample was taken from the tail vein at TP 0 min. After 90 min, mice were granted ad libitum access to regular chow food for 2 hr. The final blood sample (TP 210 min) was obtained by tail.

Analytic Procedures

Prior to tissue analyzes, animals were anesthetized and killed and their tissues were snap frozen in liquid nitrogen. Blood was collected in EDTA tubes, and glucose was measured by Freestyle Freedom glucose analyzer (Abbot, Abott Park, IL). We analyzed glucose fluxes and Ra glycerol as described elsewhere (Kang et al., 2007; Liu et al., 1998; Obici et al., 2002; Pocai et al., 2005). Plasma metabolite assays, WAT NE measurements, and tracer methodology details are described in Supplemental Experimental Procedures.

FAS Activity

FAS activity in perirenal fat was measured spectrophotometrically using a modified protocol of Nepokroeff et al. (1975). Detailed description can be found in Supplemental Experimental Procedures.

Triglyceride Hydrolase Activity

Assay was performed with epididymal fat pads as previously described (Schweiger et al., 2006). Cytosolic and lipid droplet fraction from rat adipose tissue were obtained using a modified protocol of Schweiger et al. (2008). Detailed description can be found in Supplemental Experimental Procedures.

Fatty Acid Analysis

Fatty acid analysis was performed as previously described (Scheja et al., 2008). Detailed description can be found in Supplemental Experimental Procedures.

RNA Extraction and Quantitative Real-Time RT-PCR

Detailed description and forward and reverse customized primer pairs (Invitrogen, Carlsbad, CA) are described in Supplemental Experimental Procedures.

Western Blot Analyses

Western blots were performed as previously described (Buettner et al., 2008). Detailed description and antibodies used can be found in Supplemental Experimental Procedures.

Statistics

All data are represented as mean ± SEM. Comparisons among groups were made using one-way ANOVA followed by unpaired two-tailed Student's t tests if not otherwise indicated. Differences were considered statistically significant at $p < 0.05$. For Figures 2F, 6D, S2H, and S2I, we used Pearson correlation and a two-tailed t test performed in GraphPad Prism 5.0b for Mac (GraphPad Software, San Diego, CA).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, five figures, and one table and can be found with this article online at doi:10.1016/j.cmet.2011.01.008.

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