Mediobasal hypothalamic PTEN modulates hepatic insulin resistance independently of food intake in rats

Takashi Sumita,¹ Hiraku Ono,¹ Suzuki T, Sakai G, Inukai K, Katagiri H, Asano T, Katayama S, Awata T. Mediobasal hypothalamic PTEN modulates hepatic insulin resistance independently of food intake in rats. Am J Physiol Endocrinol Metab 307: E47–E60, 2014. First published May 13, 2014; doi:10.1152/ajpendo.00361.2013.—Phosphatase and tensin homolog (PTEN) dephosphorylates phosphatidylinositol (PI) 3,4,5-triphosphate and antagonizes PI 3-kinase. Insulin acts in the mediobasal hypothalamus (MBH) to not only suppress food intake and weight gain but also improve glucose metabolism via PI 3-kinase activation. Thus, the blocking of hypothalamic PTEN is a potential target for treating obesity as well as diabetes. However, genetic modification of PTEN in specific neuronal populations in the MBH yielded complex results, and no postnatal intervention for hypothalamic PTEN has been reported yet. To elucidate how postnatal modification of hypothalamic PTEN influences food intake as well as glucose metabolism, we bidirectionally altered PTEN activity in the MBH of rats by adeno viral gene delivery. Inhibition of MBH PTEN activity reduced food intake and weight gain, whereas constitutive activation of MBH PTEN tended to induce the opposite effects. Interestingly, the effects of MBH PTEN intervention on food intake and body weight were blunted by high-fat feeding. However, MBH PTEN blockade improved hepatic insulin sensitivity even under high-fat-fed conditions. On the other hand, constitutive activation of MBH PTEN induced hepatic insulin resistance. Hepatic Akt phosphorylation and the G6Pase expression level were modulated bidirectionally by MBH PTEN intervention. These results demonstrate that PTEN in the MBH regulates hepatic insulin sensitivity independently of the effects on food intake and weight gain. Therefore, hypothalamic PTEN is a promising target for treating insulin resistance even in states of overnutrition.

Address for reprint requests and other correspondence: H. Ono, Dept. of Endocrinology and Diabetes, School of Medicine, Saitama Medical University, Moroyama, Saitama, Japan; E-mail: hono@saitama-med.ac.jp.

NUMBERS OF PEOPLE SUFFERING FROM OBESITY and type 2 diabetes are increasing worldwide. Both diseases are closely associated with insulin resistance. In peripheral insulin-sensitive tissues, insulin controls glucose metabolism mainly via activation of phosphatidylinositol 3-kinase (PI3K) (49). When stimulated with insulin, PI3K produces phosphatidylinositol 3,4,5-triphosphosphate (PIP3) in the plasma membrane. Then, PIP3 initiates the downstream signal cascade, ultimately leading to enhanced glucose uptake into muscle and adipose tissue and the inhibition of hepatic glucose production. Phosphatase and tensin homolog (PTEN) deleted on chromosome 10 is a unique dual-specific phosphatase known to dephosphorylate PIP3 and antagonize PI3K (23). Thus, PTEN has been suggested to be a potential therapeutic target, as inhibition of PTEN may ameliorate insulin resistance and diabetes. In fact, genetic as well as postnatal PTEN inhibition in muscle (55), adipose tissue (8, 20), and the liver (8, 48) have been reported to enhance insulin sensitivity.

Recently, the brain has been recognized as a new insulin target regulating energy homeostasis and glucose metabolism. Intracerebroventricular (icv) injection of insulin has been shown to suppress food intake (56). Mice lacking neuronal insulin receptors are obese and insulin resistant (7). Moreover, central nervous system (CNS) insulin indirectly improves peripheral insulin sensitivity via several mechanisms (32, 39, 42, 45). Using a site-specific stereotaxic approach and neuronal promoter-specific genetic mouse models, the mediobasal hypothalamus (MBH) containing mainly the arcuate nucleus was revealed to be one of the most important sites in the brain where insulin mediates energy homeostasis and glucose metabolism (4, 11, 40, 45, 46).

As in peripheral insulin-sensitive tissues, PI3K is reported to play a major role in the hypothalamus. Not only insulin but also leptin was shown to stimulate PI3K and/or increase PIP3 in the hypothalamus (57). Hypothalamic PI3K activation was revealed to suppress food intake and weight gain in studies using PI3K inhibitor injection into the cerebral ventricles (30, 31) as well as by genetic modification of PI3K (15). PTEN, the major antagonist of PI3K, is thus speculated to be a therapeutic target even in the CNS, where inhibition may ameliorate obesity as well as insulin resistance. However, data from genetic interventions targeting hypothalamic PTEN using the Cre-loxP system are not always consistent with this hypothesis (52). Most unexpectedly, PTEN deletion in proopiomelanocortin (POMC) neurons in the MBH paradoxically induced hyperphagia and diet-induced obesity (37). PTEN deletion in leptin receptor-expressing neurons induces leanness with fat transdifferentiation without significantly altering food intakes (38). On the other hand, overexpression of wild-type PTEN in leptin receptor-expressing neurons induced fatty liver and modestly decreased food intake without affecting adiposity or hepatic insulin signaling (54). Since these studies used promoterspecific genetic PTEN modification, they had the advantage of allowing investigation of the roles of PTEN in specific neuronal populations, but their phenotypes might also have been influenced by developmental effects. In fact, other reports have suggested that genetic deletion of PTEN in the hypothalamus leads to growth defects (9, 28) and that PTEN plays major roles in neural development (21). Moreover, since the hypothalamus is comprised of multiple cell types, the total effect of PTEN
modulation, which would more closely mimic pharmacological treatment, cannot be evaluated employing neuronal population-specific knockout treatment. Therefore, to ascertain whether hypothalamic PTEN is a potential therapeutic target for treating obesity and diabetes, it is important to investigate how postnatal PTEN intervention in mature individuals influences energy metabolism. To date, to our knowledge no postnatal experimental intervention targeting hypothalamic PTEN in adult animals has been reported. In the experiments described herein, we injected adenoviral vectors expressing dominant-negative and constitutively active PTEN mutants into the MBH to bidirectionally alter PTEN activity postnatally in mature rats and thereby investigated the functions of PTEN in this important brain region.

METHODS

Animal preparation. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Saitama Medical University. We used male Sprague-Dawley rats weighing 270–320 g (Tokyo Laboratory Animals Science). The rats were housed in individual cages, subjected to a standard light-dark cycle, and given normal chow (3.59 kcal/g; Oriental Yeast). In the high-fat diet experiments, normal chow was discontinued after virus injection and substituted with a high-fat diet (normal chow plus 10% lard, 4.85 kcal/g).

Generation of recombinant adenoviruses. Recombinant adenovirus containing myr-WT PTEN and myr-C124S PTEN cDNA of *Rattus norvegicus* was prepared by homologous recombination of expression cosmid cassettes containing the corresponding cDNAs and the parental adenovirus genome, as described previously (33). LacZ adenovirus was used as a control. The amplified adenoviruses were purified and concentrated using cesium chloride ultracentrifugation. The resultant viruses were then dialyzed against phosphate-buffered saline with 10% glycerol. Adenoviral titers were measured by an end-point dilution assay with 1:3 serial dilutions on a 96-well plate of human embryonic kidney-293 cells.

Adenovirus microinjection. The method for virus microinjection into the MBH was based on our previous report (34), with minor modifications. Using stereotactic surgery performed under gas anesthesia with isoflurane inhalation, rats were injected in the bilateral MBH (coordinates from bregma: A/P −3.0 mm, D/V −10.2 mm; Fig. 1A) with adenovirus expressing 2.7 × 10^10^ plaque-forming units per side of constitutively active PTEN (CA-PTEN), dominant negative PTEN (DN-PTEN), or LacZ. Adenovirus was delivered at a rate of 0.1 µl/min for 20 min (2 µl/injection site), and the injector and cannula were removed 40 min after completion of the injections. Body weights and food intakes were monitored from 3 to 6 days before to 7 to 14 days after virus administration. In the high-fat diet experiment, rats were shifted from a normal chow to a high-fat diet immediately after the surgery. All rats were euthanized by decapitation, and the MBH was dissected. The hypothalamic section containing the MBH was 2–4 mm posterior to the optic chiasm, a site from which we collected an equilateral triangle, with the height defined as one-third of the ventral side of the third ventricle, including the median eminence.

Plasma insulin tolerance test. Plasma insulin concentrations were measured with the Merckodia Ultrasensitive Rat Insulin ELISA kit in the food intake study and the Merckodia Rat/ Human Insulin ELISA kit in the clamp study according to the manufacturer’s protocols. Insulin sensitivity was measured by insulin tolerance test following a 5-h fast. This study was performed 8 days after the adenoviral injections. Following an intraperitoneal injection of insulin (Novolin R; Novo Nordisk, Denmark), blood glucose levels were determined at t = 0, 15, 30, and 45 min using a hand-held glucose analyzer (Sanwa Kagaku Kenkyusho).

**Tissue processing and Western blot analysis.** Brain blocks were dissected. The hypothalamic PTEN MODULATES HEPATIC INSULIN SENSITIVITY

Tissue blocks were set to 5 µm and stained with 4%, paraformaldehyde. After 2 h of fixation, the brain blocks were cryoprotected with 20% sucrose, frozen, and then sliced into coronal sections 40 µm in thickness. LacZ staining was performed with a LacZ staining kit according to the manufacturer’s instructions, followed by counterstaining with neutral red. Sections for immunofluorescence were blocked with 3% goat or donkey serum and incubated overnight at 4°C with mouse anti-LacZ (Sigma-Aldrich, 1:300), mouse anti-PTEN (Sigma-Aldrich, 1:100), or rabbit anti-LacZ (1:3,000; MP Biochemical) antibodies and then with rabbit anti-mouse secondary antibody conjugated with Alexa Fluor 488 or anti-rabbit antibody conjugated with Alexa Fluor 633 (Life Technologies). The sections were washed and then incubated with NeuroTrace 640/660 (1:300; Life Technologies). Rabbit anti-mouse IgG-HRP (1:100; Phoenix Pharmaceuticals), rabbit anti-goat-related protein (AgRP; 1:100; R & D Systems, mouse anti-glial fibrillary acidic protein (GFAP) (1:100; Sigma-Aldrich), or rabbit anti-Iba1 (Wako, 1:100) antibodies for 1 h at room temperature. The sections incubated with second primary antibodies were then incubated with anti-rabbit antibody conjugated with Alexa Fluor 633 (POMC, AgRP, Iba1) or anti-mouse antibody conjugated with Alexa Fluor 488 (GFAP). The images were captured employing a confocal microscope LSM 700 (Carl Zeiss). For quantitative analysis of adenos viral expression, three coronal sections of the brain, harboring needle scars, were selected from each animal and immunostained. Then, NeuroTrace, POMC, or AgRP-positive cells (total ≥200 cells/animal) were counted manually in a 200 × 200 µm square field in the MBH of each section, and the percentages of LacZ or PTEN colocalization with each of the neuronal markers were calculated as reported previously (22).

**RNA preparation and real-time PCR.** Total RNA was isolated from frozen liver and MBH wedges using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was quantified using a NanoDrop 1000 (NanoDrop Products). Following treatment with DNase I, purified RNA was used as a template for first-strand cDNA synthesis using SuperScript III (Life Technologies). Quantitative real-time RT-PCR was run using ABI PRISM Model 7000 (Applied Biosystems) according to the manufacturer’s instructions. The Taqman probes for rat GAPDH (Rn99999916_s1), glucose-6-phosphatase (G6Pase; Rn00565347_m1), phosphoenolpyruvate carboxykinase (PEPCK; Rn01529009_g1), fatty acid synthase (FAS; Rn01463550_m1),...
and acetyl-CoA carboxylase (ACC; Rn00573474_m1) were purchased from Applied Biosystems. Primer sets for peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α; 5'-AAAGGGCCAAGCAAGAGAGA and 5'-GTAAATCACACGGCGCTCTT) and glucokinase (GK; 5'-TATGAAGACCGCCAATGTGA and 5'-TTCCCACCGATGATCTTCTC) were used for quantitative PCR employing SYBR Green. Primer sets for Il6, TNFα, Ikbkb, Ikbke, GFAP, Emr1, and CD68 were created as described in a previous report (51).

Hyperinsulinemic euglycemic clamp. After 1 wk of recovery from the stereotaxic surgery (Fig. 5A), animals were implanted with poly-
urethane catheters (MRE025; Braintree Scientific) in the left carotid artery for sampling and the right jugular vein for infusion under gas-anesthesia with isoflurane inhalation. The catheters were tunneled subcutaneously and exteriorized at the back of the neck. The clamp study was performed 1 wk later (Fig. 5, A and B) under conscious and unrestrained conditions, as in our previous study (34). A primed, continuous infusion of \([3-\text{H}]\)glucose (40 \(\mu\)Ci bolus, 0.4 \(\mu\)Ci/min; Muramachi Pharmaceutical) was started and maintained for 2 h as the basal period. A pancreatic insulin clamp with an infusion of 2.5 \(\mu\)U/kg \(\cdot\) min \(^{-1}\) insulin and 3 \(\mu\)g/kg \(\cdot\) min \(^{-1}\) somatostatin was then initiated at \(t = 0\) and lasted for 2 h. During this clamp period, 20% glucose solution was infused at a variable rate to maintain plasma glucose concentrations within the range of \(\sim 80–110\) mg/dl. Samples for determination of \([3-\text{H}]\)glucose-specific activity were obtained at \(t = -30, -15, 0, 80, 90, 100, 110,\) and 120 min from the start of the clamp period, deproteinized with \(\text{Ba(OH}_2\) and \(\text{ZnSO}_4\), and finally dried. Samples for measurements of hormonal factors were taken at \(t = 0, 100,\) and 120 min. At the end of the clamp studies, rats were euthanized with pentobarbital sodium and tissue samples freeze-clamped in situ with steel tongs precooled in liquid nitrogen. All tissue samples were stored at \(-80^\circ\)C for subsequent analysis.

**Statistical analysis.** Statistical analyses were performed using Graphpad Prism version 6 and S-Plus version 8. Body weight change and food intake data were analyzed using a mixed-effects model in which the random effect is animal ID and the fixed effects are MBH intervention and postoperative days. Other data were analyzed using ANOVA followed by multiple comparisons employing Holm-Sidak’s post hoc test. The LacZ group was used as a control for the multiple comparisons. Multiple comparisons for the clamp study were performed for three pairs [LacZ-injected animals fed normal chow (LacZ-NC) vs. CA-PTEN-NC, LacZ-NC vs. LacZ-HF, and LacZ-HF vs. DN-PTEN-HF].

**RESULTS**

**PTEN blockade in the MBH reduced food intake and weight gain in rats.** To investigate functions of PTEN in the MBH, we injected adeno viral vectors expressing constitutively active PTEN (myr-PTEN; CA-PTEN), dominant-negative PTEN (myr-C124S-PTEN; DN-PTEN), or LacZ (control) using stereotoxic surgery (Fig. 1A). To validate our stereotoxic targeting technique, we confirmed the presence of \(\beta\)-galactosidase expression with a specific stain for LacZ 2 wk after the LacZ virus injection. As shown in Fig. 1B, \(\beta\)-galactosidase expression was successfully localized to the MBH. We also stained coronal sections of rat brain for LacZ 3 days after the LacZ virus injection. As shown in Fig. 1C, a blue color in response to LacZ staining indicates successful localization to the MBH. To determine which cell type(s) had been infected by the adeno viral vectors, we stained the brain sections with LacZ or overexpressed PTEN with each of the neuronal markers were quantitated. As shown in Fig. 2, LacZ expression or PTEN overexpression was observed as high intensity of green immunofluorescence in some (21–35%) cells in the MBH (Fig. 2, D and E), allowing discriminiation from the endogenous PTEN expression in adjacent cells that showed much lower levels of green immunofluorescence. As shown in Fig. 2D, PTEN immunofluorescence was colocalized with NeuroTrace 640/660 as well as POMC and AgRP, similar to that of LacZ (Fig. 1D). To confirm that the neuronal subpopulations affected by viral vectors did not differ among vectors, the colocalizations of LacZ or overexpressed PTEN with each of the neuronal markers were quantitated. As shown in Fig. 2E, LacZ expression or PTEN overexpression was colocalized in 21–33% of NeuroTrace-positive, 31–35% of POMC-positive, and 27–36% of AgRP-positive cells. Two-way ANOVA showed no significant differences in the colocalization percentages either between LacZ and PTEN or among the neuronal markers.

Daily food intake and weight gain before and after the viral injections were measured, and we detected a significant suppression of food intake in response to DN-PTEN expression in the MBH compared with the other groups from postoperative days 1 to 3 (LacZ 13.7 \(\pm\) 1.9 g, CA-PTEN 14.7 \(\pm\) 2.0, DN-PTEN 8.5 \(\pm\) 1.8, \(P = 0.049\), between LacZ and DN-PTEN; Fig. 2F). Weight gain from the day before the injection was also significantly lower in the DN-PTEN group (means \(\pm\) SE on postoperative days 1–6 were as follows: LacZ –22.6 \(\pm\) 2.4 g, CA-PTEN –17.1 \(\pm\) 1.9 g, DN-PTEN –42.1 \(\pm\) 1.4 g, \(P = 0.022\) between LacZ and DN-PTEN; Fig. 2G). The effects on food intake and body weight change of DN-PTEN injection were observed from day 1, i.e., in parallel with the rapid protein expression induced by adeno viral injection (Fig. 2C). The difference in body weight gain between the LacZ and DN-PTEN groups became smaller after day 7, also paralleling the decrease in protein expression after day 7 (Fig. 2C). On the other hand, when CA-PTEN was expressed in the MBH, rats gained significantly more weight than the control group (means \(\pm\) SE on postoperative days 7–10 were as follows: LacZ –9.7 \(\pm\) 2.7 g, CA-PTEN 0.9 \(\pm\) 2.5, DN-PTEN –25.0 \(\pm\) 2.3, \(P = 0.048\) between LacZ and CA-PTEN, \(P = 0.021\) between LacZ and DN-PTEN; Fig. 2G). Food intake of CA-PTEN animals tended to be higher than that in the LacZ group (\(P = 0.09\) on postoperative day 4; Fig. 2F). The relatively late
Fig. 2. Phosphatase and tensin homolog (PTEN) blockade in the MBH suppressed food intake and weight gain in rats. A: DNA constructs of viruses. Constitutively active (CA)-PTEN, wild-type PTEN with myristoylation signal sequence and FLAG tag added to the NH2-terminal; dominant negative (DN)-PTEN, construct with cysteine 124 mutated to serine, known to be a catalytic-dead mutant, with myristoylation signal and FLAG tag. B: the expressions of CA-PTEN and DN-PTEN in the MBH were confirmed by immunoblotting using anti-PTEN antibody. Results are shown as representative bands. C: time course of exogenous PTEN expression in the MBH. MBH lysates were immunoprecipitated using M2-Flag affinity gel and immunoblotted employing PTEN antibody. Results are shown as representative bands and quantification of all bands. Multiple comparisons (quantification results on day 1 served as the control for Holm-Sidak’s test) were carried out after ANOVA. D: cell types with overexpression of PTEN mutants confirmed by double immunostaining of PTEN (green) immunofluorescence and NeuroTrace 640/660 or antibodies against POMC or AgRP (red). Merged images are shown. Arrows point to the cells in which overexpressed PTEN and the neuronal marker are colocalized. CA, CA-PTEN; DN, DN-PTEN. E: quantification of MBH LacZ- or PTEN-overexpressing neurons. LacZ-positive (see Fig. 1D) or PTEN-overexpressing (D) cells (green) colocalized with NeuroTrace 640/660 or with immunofluorescence of POMC or AgRP (red) were counted and expressed as percentages of the no. of each respective neuronal marker-positive cell. N, NeuroTrace 640/660; P, POMC; A, AgRP (n = 4 in each group). F: mean daily food intakes of LacZ, CA-PTEN, or DN-PTEN rats on normal chow. G: mean daily body weights, shown as the difference from the day of the MBH injection. ○, LacZ; ▲, CA-PTEN; ■, DN-PTEN (n = 7, 8, and 6, respectively). Statistical analyses were performed using a mixed-effects model in which the fixed effects are the MBH intervention and postoperative days and the random effect is animal ID, with the LacZ group as the control. Data are presented as means ± SE. *P < 0.05; **P < 0.01.
appearance of the effect of CA-PTEN on body weight might be attributable to the nonspecific body weight decreases induced by the viral infection in all of the animal groups, masking the difference between CA-PTEN and LacZ before day 7.

The injections of viral vectors induced significant reductions in food intake and body weight in all experimental animal groups. Adenoviral injection presumably induces inflammation in the MBH, and recent reports have described diet-induced hypothalamic inflammation (50, 51). To evaluate whether adenovirus-induced inflammation is related to the reductions in food intake and body weight gain, we injected LacZ adenovirus or vehicle into the MBH and measured food intakes and body weight gains. Compared with the vehicle, LacZ adenovirus markedly reduced food intake (during postoperative days 1–14; LacZ: 20.8 ± 0.7 g, vehicle: 28.6 ± 0.4 g, P < 0.001; Fig. 3A) and body weight gain (LacZ: −11.3 ± 2.6 g, vehicle: 37.6 ± 3.6 g, P < 0.001; Fig. 3B). Also, expressions of markers of inflammation such as TNFα, IL-6, and IKKe (3.5 ± 0.8-fold increase compared with vehicle, 12.3 ± 5.8, 3.5 ± 0.3, P < 0.05; Fig. 3, C, D, and F, respectively) as well as the microglia-specific marker CD68 (2.9 ± 0.4-fold, P < 0.05; Fig. 3F) were significantly elevated in the MBH by adenoviral injection compared with the vehicle. However, the levels of these markers did not differ significantly among the LacZ, CA-PTEN, and DN-PTEN groups. These observations show that adenoviral injection produces an inflammatory response in the MBH, which may contribute to postoperative suppressions of food intake and body weight, regardless of the construct in the vectors. In this study, the viral titers were matched to eliminate biased interference from inflammation, which validates our current comparisons among the LacZ, CA-PTEN, and DN-PTEN groups.

The suppressive effect of MBH PTEN intervention on food intake was blunted by high-fat feeding. Overfeeding reportedly induces resistance to insulin and leptin (53). Thus, we next examined whether overfeeding modifies the effect of PTEN intervention in the MBH. When the rats were fed a high-fat diet (normal chow mixed with 10% lard) after adenoviral injection into the MBH, the effect of DN-PTEN on food intake became minimal (Fig. 4A), and weight gain after the intervention did not differ significantly from that of control rats (Fig. 4B).

Several reports have suggested that hypothalamic insulin as well as leptin may regulate energy balance and glucose metabolism independently of each other (2, 5, 11, 19). To assess insulin sensitivity, we performed insulin tolerance tests in the three experimental animal groups on postoperative day 8, when the body weights of the animals had recovered to >95% of their original levels. Although body weights did not differ significantly among the groups after high-fat feeding, the ability of insulin to reduce blood glucose levels was significantly more potent in DN-PTEN rats at 15 min after insulin injection (LacZ: 90 ± 3% of the glucose level at t = 0; CA-PTEN: 92 ± 6; DN-PTEN: 73 ± 6; P < 0.05 between DN-PTEN and LacZ; Fig. 4C). The fasting plasma insulin level of CA-PTEN rats was higher than that of the control group (LacZ: 0.29 ± 0.02 ng/ml; CA-PTEN: 0.59 ± 0.14; DN-PTEN: 0.30 ± 0.04; P < 0.05 between DN-PTEN and LacZ; Fig. 4D). These results indicate that inhibition of MBH PTEN enhances insulin sensitivity and that overactivation of MBH PTEN may induce insulin resistance.

A recent report demonstrated that genetic overexpression of PTEN in leptin receptor-positive neurons enhances lipogenesis in the liver (54). Thus, we investigated whether hepatic lipogenic genes are affected by our PTEN modulations in the MBH. In the high-fat-fed state, hepatic expressions of two major lipogenic genes, FAS and ACC, are increased significantly in CA-PTEN rats (3.3 ± 1.2- and 1.7 ± 0.4-fold, respectively, P < 0.05; Fig. 4, E and F), which is consistent with the findings in a previous report (54). Liver triglyceride levels did not differ significantly among the three groups (Fig. 4G).

Bidirectional MBH PTEN intervention revealed its regulatory role in endogenous glucose production. Since insulin tolerance test suggested MBH DN-PTEN to enhance insulin sensitivity and increased fasting plasma insulin level suggested CA-PTEN to impair it, we subsequently carried out a hyperinsulinemic euglycemic clamp study with conscious, unrestrained rats to analyze in detail the effects of MBH PTEN intervention on insulin sensitivity. To eliminate interference from endogenous insulin and glucagon as well as from counterregulatory hormonal secretions driven by hypoglycemia, somatostatin was infused continuously, and the glucose infusion rate was adjusted to maintain euglycemia during the clamp period. Rats were divided into four groups: MBH LacZ with normal chow (LZ-NC), MBH CA-PTEN with normal chow (CA-PTEN-NC), MBH LacZ with high-fat diet (LZ-HF), and MBH DN-PTEN with high-fat diet (DN-PTEN-HF). As described above, when fed normal chow, food intakes of CA-PTEN animals tended to be higher than that of the LacZ group. Therefore, to eliminate the effects on insulin sensitivity of any tendency for an increase in food intake associated with MBH CA-PTEN expression, we measured the food intake of each animal in the chow-fed LacZ group on days 1–7 and provided an identical amount of food to the paired animals in the CA-PTEN group. After vascular surgery, food was not restricted. There were no significant differences in body weight between LZ-NC and CA-PTEN-NC rats or between LZ-HF and DN-PTEN-HF rats (Table 1). Humoral factors such as NEFA, insulin, corticosterone, and triglycerides did not differ significantly among the experimental groups, except for higher serum triglyceride levels during the clamp period in the MBH CA-PTEN-expressing rats (Table 1).

During the last 30 min of the basal period, blood glucose was lower in the DN-PTEN-HF group (Fig. 5C). To eliminate experimental biases, we used a fixed glucose infusion rate for...
the first 30 min of the clamp period in all animal groups and adjusted it to maintain euglycemia thereafter. Thirty minutes after the start of the clamp period, blood glucose was lower in the LacZ-NC and DN-PTEN-HF than in the other two groups (Fig. 5C), suggesting insulin sensitivities to be higher in the former than in the latter two groups. The glucose infusion rate (GIR) required for maintaining euglycemia was significantly lower in the CA-PTEN-NC group (3.7 ± 0.2 mg·kg\(^{-1}\)·min\(^{-1}\), \(P < 0.05\)) as well as the LacZ-HF group (2.4 ± 1.0 mg·kg\(^{-1}\)·min\(^{-1}\), \(P < 0.01\)), than in the LacZ-NC group (10.8 ± 1.6 mg·kg\(^{-1}\)·min\(^{-1}\)) (Fig. 5D). Conversely, GIR in the DN-PTEN-HF group (10.5 ± 2.0) was significantly higher (\(P < 0.05\)) than that in the LacZ-HF group. Tracer analyses revealed that endogenous glucose production (EGP) during the clamp period was significantly elevated by MBH CA-PTEN expression (9.2 ± 1.0 mg·kg\(^{-1}\)·min\(^{-1}\)) as well as high-fat diet feeding blunted the effects of MBH PTEN on food intake, whereas those on insulin sensitivity and hepatic lipogenesis persisted. The food provided to rats (\(n = 6\) in each group) was switched from normal chow to a high-fat diet after adenovirus injection. A: mean daily food intake. B: body weight change. C: insulin tolerance tests done on postoperative day 8. Five-hour-fasted rats were injected intraperitoneally with insulin (1.5 Ukg). D: plasma insulin concentration after a 5-h fast on postoperative day 9. E and F: liver fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) expressions were determined by real-time PCR. mRNA levels were normalized to GAPDH. The graphs are presented as the fold increase over LacZ. G: liver triglyceride content. Data are presented as means ± SE. Multiple comparisons (LacZ served as the control for Holm-Sidak’s test) were carried out after ANOVA. *\(P < 0.05\) between groups.
feeding (9.0 ± 1.1) compared with the control group (4.8 ± 0.7 mg·kg⁻¹·min⁻¹, P < 0.05; Fig. 5E). On the other hand, EGP during the clamp period was significantly reduced by MBH DN-PTEN expression (3.5 ± 1.5 mg·kg⁻¹·min⁻¹) compared with the LacZ-HF group (P < 0.05; Fig. 5E). As reported previously (53), high-fat diet feeding induced higher EGP (Fig. 5E) and lower glucose uptake (LacZ-NC: 15.5 ± 0.7; P < 0.01; Fig. 5F). MBH PTEN intervention did not change peripheral glucose uptake significantly (Fig. 5F).

**Bidirectional MBH PTEN intervention modulated hepatic akt phosphorylation and G6Pase expression.** Finally, we analyzed the liver samples that had been snap-frozen just after the clamp study. Akt phosphorylation at Ser473 was blunted by MBH CA-PTEN expression (0.50 ± 0.09-fold) as well as by high-fat diet feeding (0.57 ± 0.07-fold; Fig. 6A). Conversely, MBH inhibition of PTEN by DN-PTEN expression reversed this high-fat diet-induced blunting of Akt phosphorylation (0.57 ± 0.07 vs. 1.02 ± 0.18-fold; Fig. 6A). Regarding another potential signal mediator of CNS insulin for hepatic glucose production (16), STAT3, the phosphorylation of which at Tyr705 tends to be increased peripheral glucose uptake significantly (Fig. 5F).

**DISCUSSION**

When PTEN was blocked in peripheral insulin target tissues, enhancement of insulin sensitivity was observed (8, 20, 48, 55). However, genetic PTEN intervention in hypothalamic neurons has shown much more complex results in previous studies. Partial PTEN deletion in the hypothalamus using RIP-Cre resulted in whole body growth restriction (9, 28). PTEN deletion in POMC-expressing neurons paradoxically resulted in hyperphagia and diet-induced obesity (37). Surprisingly, PI3K deletion in the same POMC neurons was also reported to increase body weight (1, 15). PTEN deletion in leptin receptor-expressing cells led to leanness due to increased energy expenditure, with no significant suppression of food intake (38). PTEN overexpression in the same leptin receptor-expressing cells induced liver steatosis without obesity or any form of increased adiposity, instead producing a modest decrease in food intake (54). Additionally, both PTEN deletion (18) and PI3K deletion (58) in ventral medial hypothalamic SF-1-positive neurons led to diet-induced obesity. These seemingly conflicting results may reflect the complexity of the hypothalamus, where various types of neurons, sometimes with opposite functions, exist in proximity to each other. Also, several outputs such as transcriptional regulation and electrophysiological activity can exist even in a single neuron, which may result in complex effects. Moreover, these apparently inconsistent results may have arisen at least in part from developmental effects of PTEN deletion during the prenatal period, given that PTEN has been well established as being important, especially in neural development (13, 47), and that POMC is broadly expressed in immature hypothalamic neurons during the gestational period (35, 36). Interestingly, to our knowledge, no study to date has shown that PTEN inhibition in the hypothalamus suppresses food intake or that PTEN activation increases it, which would be expected based on the theory that PI3K and PIP3 are negative regulators of food intake.

Herein, we investigated how bidirectional postnatal PTEN intervention in the MBH of mature animals affects food intake, body weight gain, and insulin sensitivity. Our approach is anatomically specific to the MBH, where the arcuate nucleus is...
located. Also, the adenoviral vector containing the CMV early enhancer/chicken β-actin promoter-driven cassette was shown to express the constructed protein mainly in neurons, with little or no expression in astrocytes or microglia in the MBH. However, the vectors used in this study have no specificity for any of the neuronal populations in the MBH. Thus, we speculate that the outcomes obtained represent the net effects of PTEN intervention in all types of neurons in the MBH, which would more closely mimic pharmacological treatment in adult individuals than the results obtained with promoter-specific genetic mouse models. First, we found that MBH PTEN inhibition induces significant suppressions of both food intake and body weight gain and that MBH PTEN activation tends to exert the opposite effects. These results are consistent with the theory that PIP3 plays a second-messenger role for insulin and leptin in the arcuate nucleus, promoting the suppression of food intake and weight gain, and that endogenous PTEN plays a role in degrading hypothalamic PIP3. Our results are also

Fig. 5. Hyperinsulinemic euglycemic clamp study of rats with bidirectional modification of MBH PTEN. A: protocols for surgeries, viral injection, and the insulin clamp study. B: clamp protocol. C: plasma glucose levels during the basal and clamp periods. □, Chow-fed LacZ; ◊, chow-fed CA-PTEN; ■, high-fat diet (HFD)-fed LacZ; ▲, HFD-fed DN-PTEN (n = 5). D: glucose infusion rate (GIR) required to maintain euglycemia during the clamp period. Bars show average GIR during the last 40 min of the clamp period. E: endogenous glucose production (EGP) during the basal and clamp periods. F: peripheral glucose uptake during clamp period. Data are presented as means ± SE. Multiple comparisons were performed for 3 pairs (LacZ-NC vs. CA-PTEN-NC, LacZ-NC vs. LacZ-HF, and LacZ-HF vs. DN-PTEN-HF) employing Holm-Sidak’s test after ANOVA. *P < 0.05; **P < 0.01. Rd, glucose disappearance.
Fig. 6. Analysis of liver samples after the hyperinsulinemic euglycemic clamp study. A and B: quantification of hepatic Akt phosphorylation at Ser473 (A) and STAT3 phosphorylation at Tyr705 (B). A and B, top: representative bands from each group. A and B, bottom: ratio of phosphoproteins to total proteins. C–H: hepatic glucose-6-phosphatase (G6Pase; C), phosphoenolpyruvate carboxykinase (PEPCK; D), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α; E), glucokinase (GK; F), FAS (G), and ACC (H) expressions were determined by real time-PCR. mRNA levels were normalized to GAPDH. The graphs are presented as the fold increase over the chow-fed LacZ group. I: liver triglyceride (TG) content after the clamp study. Data are presented as means ± SE; n = 5. Multiple comparisons were performed for 3 pairs (LacZ-NC vs. CA-PTEN-NC, LacZ-NC vs. LacZ-HF, and LacZ-HF vs. DN-PTEN-HF), with Holm-Sidak’s test employed after ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.
consistent with results obtained by altering PI3K in POMC neurons (1, 15) and with those resulting from pharmacological inhibition of PI3K in the CNS (30, 31) and adenoviral intervention aimed at the insulin/leptin signaling in the MBH (11, 25). However, our observations are inconsistent with those reported for genetic deletion of PTEN in POMC neurons (37). The reason for this inconsistency is not clear, but it may indicate that the influence of PTEN on food intake is predominantly in a cell population other than POMC neurons in the MBH, as described by the authors of the aforementioned report (37). Alternatively, it is also possible that genetic deletion of PTEN induces other effects in addition to simply increasing PIP3 (52).

Unexpectedly, the effects on food intake and body weight were blunted when the animals were fed a moderately (normal chow plus 15% lard) high-fat diet. A high-fat diet is known to blunt the food intake-suppressing effect of leptin (leptin resistance). Diet-induced resistance to insulin and/or leptin reportedly occurs at the receptor or receptor substrate level (50), and our observations in this study that a high-fat diet blunted PTEN’s effects on food intake and body weight are inconsistent with these prior findings. The signaling cascade of insulin and leptin downstream from PIP3, which suppresses food intake, has not been clearly defined, whereas involvements of several pathways such as the PIP3-KATP channel interaction (24), transcriptional regulation of Foxo1 (17), and the PDE3B-cAMP pathway (44) have been reported. Although elucidating the mechanism by which high-fat feeding blunted the effects on food intake and body weight with MBH PTEN intervention would be difficult, based only on the results obtained in this study it is not unreasonable to speculate that the high-fat diet-induced leptin/insulin resistance in the MBH, as reflected by changes in food intake, occurs partly downstream from PTEN. On the other hand, preservation of the improvement in hepatic insulin sensitivity even in the high-fat diet-fed state indicates that hypothalamic insulin resistance occurs upstream from PTEN, which is consistent with previous studies showing that the insulin resistance in hypothalamic occurs at the insulin receptor substrate level (34).

Insulin has been shown to suppress hepatic glucose production indirectly via hypothalamic signaling in addition to its direct effect in the liver (32, 39, 45). Herein, we have for the first time demonstrated that targeting hypothalamic PTEN enhances this indirect pathway and may restore hepatic insulin sensitivity diminished by high-fat diet feeding. This is consistent with a previous report showing that ivc injection of a PI3K inhibitor blunted the ability of insulin to suppress hepatic glucose production (32). Among genetic hypothalamic PTEN-manipulated murine models, only one, the leptin receptor-specific PTEN-KO mouse, has been evaluated for insulin sensitivity and shown enhanced glucose uptake with no change in hepatic glucose production (38). On the other hand, enhancement of PI3K activity by deletion of its regulatory domain in POMC neurons leads to suppression of hepatic glucose production independently of body weight change (15). At the hormone receptor level, ablation of both insulin and leptin receptors in POMC neurons, as well as ablation of insulin receptors in AgRP neurons, induced hepatic insulin resistance (14, 19). Reconstitution of insulin receptors in AgRP neurons, as well as reconstitution of leptin receptors in POMC neurons, restored hepatic insulin sensitivity (5, 22). Taking these reports into consideration, our present results suggest that the net effect of PTEN inactivation in various neuronal subpopulations, including POMC and AgRP neurons in the MBH, reverses diet-induced hepatic insulin resistance. Since the blood-brain barrier is not as tight in the MBH as in other brain areas (43), our data raise the possibility of pharmacological inhibition of PTEN in the MBH being a potential treatment for hepatic insulin resistance and type 2 diabetes.

Molecular mechanisms in the liver for receiving CNS signals and regulating hepatic insulin sensitivity are not yet well understood, although IL-6/STAT3 is currently the most promising pathway (16). Herein, we have shown constitutive MBH PTEN activation as well as high-fat diet feeding to blunt hepatic Akt phosphorylation and that inhibition of MBH PTEN reverses this high-fat diet-induced blunting of Akt phosphorylation. Changes in hepatic Akt phosphorylation via CNS input were also reported previously in the POMC-specific PI3K-KO mouse model (15) and in MBH leptin receptor-reconstituted rats (12). The expression of G6Pase was affected in the direction opposite Akt phosphorylation, suggesting that MBH PTEN intervention modulates hepatic Akt activity, which regulates G6Pase expression and ultimately hepatic insulin sensitivity. STAT3 phosphorylation tended to decrease with MBH CA-PTEN expression, but the variability in phospho-STAT3 was relatively large, such that the differences among groups were not significant. This variability might be attributable to relatively slow STAT3 phosphorylation, which is reportedly much higher at 3 h after insulin stimulation than at earlier time points (16). MBH expression of CA-PTEN additionally affected other factors; serum triglycerides and FAS expression at the end of the clamp period were significantly higher in the CA-PTEN group. FAS upregulation was also reported in the POMC-specific PTEN transgenic mouse, in which liver triglyceride levels were significantly increased (54). The reason for our CA-PTEN rats showing only a tendency for increased hepatic triglycerides may be the shorter time course (2 wk) compared with the transgenic model. The serum triglyceride increase might be responsible for insulin resistance induced by CA-PTEN expression in the MBH, since hypertriglycerideremia is known to be associated with insulin resistance, and CNS insulin was recently reported to control lipolysis and lipogenesis in white adipose tissue (45). Further study is required to determine whether serum triglycerides are involved in the underlying mechanism. Another interesting finding in this study is marked downregulation of GK by MBH PTEN activation. Liver GK mRNA is reportedly increased in dogs by head artery insulin infusion (42). Since insulin is known to enhance GK transcription, the decrease in GK may be the result of hepatic insulin resistance. However, it is not clear whether this change is responsible for blunted suppression of EGP, since decreased hepatic GK has been shown to affect glucose flux only under hyperglycemic conditions (3, 41). On the other hand, suppression of MBH PTEN by DN-PTEN expression did not significantly affect serum triglycerides, hepatic FAS, or GK expression, suggesting that these are unlikely to be major mediators by which MBH PTEN suppression reverses high-fat diet-induced hepatic insulin resistance.

The major limitation of this study is the transient and nonspecific expression patterns induced by adenoviral vectors. Although injection of viral vectors into the MBH has been used in many previous studies (6, 10, 25, 26, 34, 57) and is widely
regarded as an established method, due to its nonspecificity, it is impossible to identify which neuronal subpopulation is responsible for the changes in energy and glucose metabolism obtained by using this method. Previous studies showing POMC-specific deletions of PI3K catalytic subunits to reduce body weight (1, 15), although their AgRP-specific deletions induced obesity (1), support the idea that some of our observations regarding body weight might be due to POMC neurons. Regarding hepatic insulin sensitivity, PIP3 signaling in both POMC (14, 15) and AgRP (19, 22) neurons are reportedly involved. We actually confirmed that our PTEN mutants are expressed in both POMC and AgRP neurons; i.e., it is reasonable to speculate that the change in hepatic insulin sensitivity observed in our study occurs via both of these neuronal subtypes. Future studies using methods other than just adenoviral injection, such as inducible promoters and the Cre-loxP system, will be needed to identify which neuronal subpopulation(s) is most important for PTEN modulation of glucose and energy metabolisms in mature animals.

In summary, we have shown that postnatal PTEN blockade in the MBH suppresses food intake and weight gain but that these effects are blunted by overnutrition. PTEN inhibition in the MBH ameliorates diet-induced hepatic insulin resistance independently of the effects on food intake and body weight, suggesting hypothalamic PTEN to be a potential target for treating insulin resistance and type 2 diabetes.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


