PDK-1/FoxO1 pathway in POMC neurons regulates Pomc expression and food intake

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Insulin and leptin, which are secreted from pancreatic β-cells and adipose tissues, respectively, mediate peripheral energy homeostasis signals for the hypothalamic ARC. Insulin and leptin receptors are both expressed in the ARC and have an inhibitory effect on food intake (10, 30). Several studies using genetically modified mice for insulin-signaling molecules demonstrated that insulin signaling in the ARC has important roles in regulating energy homeostasis and glucose metabolism (6, 8, 17, 18). More recently, using the Cre recombinase-mediated method, the neuron-specific roles of insulin-signaling molecules in the ARC have been shown to have selective roles in POMC or AgRP neurons (8, 16, 27, 28). On the other hand, leptin and leptin receptor in the central nervous system are important for regulation of energy homeostasis. The obese (ob/ob) mice were shown to harbor a loss-of-function mutation in leptin gene, and the phenotype of diabetic (db/db) mice is due to mutations in the “long”, or “signaling” form of the leptin receptor (LeprB) (9).

On insulin binding, the insulin receptor recruits several intracellular substrates involved in signal transduction. Among them, IRS proteins are phosphorylated by the activated insulin receptor and in turn activate PI(3)K, which generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phosphatidylinositol-4,5-bisphosphate (PIP2). PIP3-mediated activation of PDK-1 activates an enzyme cascade that includes protein kinase B (PKB, also known as Akt) and members of the atypical PKC family. On the other hand, the leptin receptor is a class 1 cytokine receptor that regulates gene transcription via activation of the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway. Leptin binds to LeprB and stimulates JAK2 to phosphorylate STAT3. Phosphorylated STAT3 dimerizes and enters the nucleus to regulate the transcription of target genes. Interestingly, leptin also activates the IRS/PI(3)K pathway (19). Therefore, both insulin and leptin signaling pathways converge on PI(3)K/PDK-1/Akt.

Among the targets of activated Akt are the mammalian target of rapamycin (mTOR) and the forkhead transcription factor O1; proopiomelanocortin neurons; leptin and leptin receptor in the central nervous system are important for regulation of energy homeostasis (3, 9, 19, 31).

Neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons, have important roles in energy homeostasis (3, 9, 19, 31).
factor FoxO1 (19). FoxO1 is a downstream element of insulin signaling; it is phosphorylated and inhibited in a PI(3)K-dependent manner (1). Recent FoxO1 studies using adenoviral delivery to the hypothalamus have demonstrated that FoxO1 increased food intake through the activation of AgRP and/or Npy gene expression and the inhibition of POMC gene expression (13, 14). Belgardt et al. (4) delineated the importance of pathways downstream of PI(3)K in POMC neurons by using mice in which Pdk1 was inactivated specifically in POMC neurons. However, the contribution of FoxO1 in the PI(3)K-PDK-1/Akt pathway or the chronic effects of overexpression of FoxO1 on regulation of energy homeostasis in POMC neurons has not been elucidated.

In the present study, we generated POMC neuron-specific Pdk1 knockout mice and transgenic mice that expressed a POMC neuron-specific constitutively nuclear or transactivation-deficient form of FoxO1 in order to examine the roles of the PDK-1 and FoxO1 in POMC neurons and discussed roles of PDK-1/FoxO1 pathway in POMC neurons.

MATERIALS AND METHODS

Transgenic mice. The pR26-1 plasmid was used to insert a conditional CNFoxO1 or Δ256FoxO1 expression cassette into the Rosa26 locus. The expression cassettes began with a splice acceptor sequence (SA), followed by Pgkneo and three polyadenylation sequences flanked by loxP sites. The CNFoxO1 or Δ256FoxO1 cDNA was followed by a bovine growth hormone polyadenylation (bpA) sequence and placed 3’ to floxed. The expression cassette, SA-loxP-Pgkneo-loxP-CN (or Δ256) FoxO1-bpA, was inserted into the PacI/AiscI sites of the pR26-1 to generate a targeting vector. A diphtheria toxin expression cassette (DT) was present within pR26-1 for negative selection. The targeting vector was electroporated into embryonic stem (ES) cells and selected in G418. The ES cell clone genomic DNA was digested with EcoRV and analyzed by Southern blotting using a 5’ external probe as described. Targeted ES cell clones were injected into C57BL/6 blastocysts to generate chimeras that transmitted the R26^flh^/CN (or Δ256) FoxO1 allele to their progeny. The R26^flh^/CN (or Δ256) FoxO1 allele was examined on a B6D12 mixed genetic background.

Breeding colonies were maintained by mating R26^flh^/CNFoxO1 or R26^flh^/CNΔ256FoxO1 mice with POMCCre mice. Animals from the same mixed-background strain generation were compared. The mice were genotyped by PCR using genomic DNA isolated from tail clippings. The primers for R26^flh^/CNFoxO1 and R26^flh^/CNΔ256FoxO1 mice were 5’-ATGGACATACAAAGACGTAC-3’ (sense) and 5’-GTGCAGGTG-GACTGTTAAAAC3’ (antisense). The primers for POMCCre mice were 5’-CAGTGGAACACTCTAGTCCGC-3’ and 5’-CAGATTAGTATATATCGGACC-3’.

Animal care. Mice were fed a standard chow diet and water ad libitum. The animals were housed in sterile cages in a barrier animal facility at 22–24°C with a 12:12-h light-dark cycle. The Animal Ethics Committee of Kobe University Graduate School of Medicine approved all experimental protocols.

Analytic procedures and tolerance tests. We carried out all assays in duplicate; each value represents the means of two independent determinations. Intrapertoneal glucose tolerance tests, insulin tolerance tests, and computed tomography (CT) scans were performed as previously described (24). The measurement of serum corticosterone was performed using the Corticosterone Correlate-EIA Kit (Assay Designs). The measurement of serum T4 was performed using the Rodent T4 ELISA Test Kit (Endocrine Technology). The measurements of serum insulin and leptin levels were performed as previously described (24).

Measurement of oxygen consumption and physical activity. Mice were monitored individually in a metabolic cage (Oxymax Windows, Columbus Instruments) with free access to a normal chow diet and drinking water for 72 h. Each cage was monitored for oxygen consumption at 10-min intervals throughout a 48-h period. Total oxygen consumption was calculated as accumulated oxygen uptake for each mouse divided by its body weight. We performed oxygen consumption of three to four mice in each genotype. For measurement of basal locomotor activity, mice were placed into chambers of a food intake, drinking, and locomotor activity monitoring system (ACTIMO-S, Shintechco). Food and water were provided ad libitum. Mice were allowed to acclimatize in the chambers for 2 h, and physical activity was measured for the following 72 h.

RNA isolation and real-time PCR. Isolation of total RNA from the hypothalamus was performed using the SV Total RNA Isolation System (Promega) according to the manufacturer’s protocol. Real-time PCR was also performed as previously described (24). The primers used in this study were described previously (24). We used the following primers for amplification of Pomp 5’-AGACGTCCATAGATTTGGAG-3’ (sense) and 5’-AGCGGAAGTACCATGACTGAGTC-3’ (antisense); AgRP 5’-AGTCGTACGTCGAATGTTGCGT-3’ (sense) and 5’-TGAGGCCCTACGACTCTAGAC-3’ (antisense); Npy 5’-AAAGCGCAACATCCGGCGGGAGG-3’ (sense) and 5’-GCTTTCCTCATAAGAGGTCTC-3’ (antisense); Cart 5’-ATCACTCCTGCCTGATAGAT-3’ (sense) and 5’-TCTTTATGCT-GACTGCTGT-3’ (antisense). Endogenous FoxO1 was amplified using FoxO1-S969 5’-GATAAGGGCCGACACAGAGG-3’ (sense) and FoxO1-A507 5’-GGCCGACAGGGTGGCATA-3’ (antisense).

Immunofluorescence. For immunofluorescence of the hypothalamus, mice were transcardially perfused with saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). The brains were dissected in 4% PFA at 4°C and soaked in 30% sucrose overnight. Free-floating coronal sections (40 μm thick) were cut from the ARC with a freezing microtome (Leica Microsystems). The sections were then extensively washed in PBS for 20 min to quench endogenous peroxidase activity. Following pretreatment, the sections were stained in 10 mM glucose. They were then incubated in HKRB supplemented with 10.2% normal goat serum (NGS) and 0.1% Tween-20 for 1 h at 37°C. Preparation of the DNA solution for ChIP was performed using a Renaissance Tyramide Signal Amplification kit (PerkinElmer) according to the manufacturer’s protocol [primary antibody Octa-Probe (D-8: sc-807), Santa Cruz Biotechnology, and mouse monoclonal (BG-02) to β-galactosidase (ab1047), abcam; secondary antibody Alexa fluoror R 555 goat anti-mouse IgG (H+L) (A21423, Molecular Probes) to double stain for FLAG and β-galactosidase.

Chromatin immunoprecipitation assay. Hypothalamic samples were trypsinized with frequent pipetting and fixed with 1% paraformaldehyde for 1 h at 37°C. Preparation of the DNA solution for ChIP PCR was performed according to the manufacturer’s directions [chromatin immunoprecipitation (ChIP) assay kit, Upstate]. Immunoprecipitation was performed using the with Octa-Probe (D-8) (sc-807, Santa Cruz Biotechnology) and anti-phospho-STAT3 (Ser/Thr) rabbit polyclonal antibody (Cell Signaling), and an equal amount of normal rabbit IgG (Santa Cruz Biotechnology). The samples were subjected to PCR using the following primers for mouse Pomp: 5’-TAAGATT-TGGGGAATCAAGGC-3’ and 5’-TCTGCCATCTCCAAGCTA-3’ (antisense).

Preparation of single ARC neurons. The ARC was excised from the brain of mice, and then, single neurons were prepared, as previously reported (15) with slight modifications. Briefly, mice were anaesthetized with urethane (ethyl carbamate, 1 g/kg ip) and decapitated, and their brains were removed. A brain slice containing the entire ARC was prepared, and the entire ARC was excised from the left and right sides. The dissected tissues were washed with 10 mM HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) containing 10 mM glucose. They were then incubated in HKRB supplemented with 20 U/ml papain (Sigma-Aldrich, St. Louis, MO), 0.015 mg/ml deoxyribonuclease (Sigma-Aldrich), 0.75 mg/ml bovine serum albumin (Sigma-Aldrich), and 1 mM cysteine for 17 min at 36°C in a shaking water bath followed by gentle mechanical trituration for 5–10
min. After trituration, the cell suspension was centrifuged at 100 g for 5 min. The pellet was resuspended in HKRB and distributed onto coverslips.

**Results**

**Generation of POMCPdk1−/−.** To investigate roles of PDK-1 in POMC neurons, we generated POMC neuron-specific Pdk1 knockout mice. For generation of POMC neuron-specific Pdk1 knockout mice, at first we crossed Pdk1flox/flox mice (12) with PomcCre transgenic mice (2) and then crossed the resulting Pdk1flox/PomcCre offspring (POMCPdk1−/) with Pdk1flox/flox mice and generated Pdk1flox/flox/POMCPdk1−/− mice. The genotypes of animals born from the latter cross confirmed to the expected ratio (data not shown). To confirm whether PDK-1 expression levels in POMC neurons were decreased, we crossed POMCPdk1−/− mice with CAG-CAT-LacZ mice (29) to generate POMCPdk1−/+;CAG-CAT-LacZ (POMCPdk1−/+;LacZ) and then crossed POMCPdk1−/−;LacZ with Pdk1flox/flox mice to generate POMCPdk1−/−;LacZ mice [Supplemental Fig. 1 (supplemental materials are found in the online version of this paper at the journal website)]. In these mice, β-galactosidase is expressed in cells where Cre-recombinase is expressed. Therefore, cells positive for β-galactosidase indicate POMC neurons in the hypothalamus. Immunohistochemistry using anti-β-galactosidase and anti-PDK-1 antibodies demonstrated that ~90% of neurons positive for β-galactosidase in POMCPdk1−/+;LacZ mice expressed PDK-1 (Fig. 1A, top). However, ~90% of neurons positive for β-galactosidase in POMCPdk1−/−;LacZ mice expressed PDK-1 (Fig. 1A, bottom). These data indicate that PDK-1 in POMC neurons of POMCPdk1−/−;LacZ mice was deleted, and we used them for the following analyses.

**Metabolic characterization of POMCPdk1−/− mice.** Body weight of both male and female POMCPdk1−/− mice was increased compared with control mice from 6 wk of age significantly (Fig. 1B). Naso-anal length (NAL) of POMCPdk1−/− mice was similar to that of control mice (data not shown). Adipose tissue mass, especially visceral fat mass, of POMCPdk1−/− mice was increased significantly compared with control mice (Fig. 1C). Total daily food intake of POMCPdk1−/− mice increased significantly (Fig. 1D). Indirect calorimetry demonstrated that oxygen consumption of POMCPdk1−/− mice was similar to that of control mice (Fig. 1E). Respiratory quotients of POMCPdk1−/− mice was similar to control mice (Fig. 1F). Locomotive activity of POMCPdk1−/− mice was similar to that of control mice (data not shown). Although serum leptin levels of POMCPdk1−/− mice were similar to those of control mice (data not shown), intraperitoneal leptin administration demonstrated that reduction of both body weight and food intake in response to leptin in POMCPdk1−/− mice was less than in control mice (Fig. 1, G and H). These data suggest that POMCPdk1−/− mice exhibit positive energy balance due to increased food intake and have leptin resistance.

Loss of PDK-1 in POMC neurons blunts influx of Ca2+ by leptin. To examine whether loss of PDK-1 affects cell number of POMC neurons, we counted cell numbers stained with anti-β-galactosidase antibody in serial hypothalamic sections from POMCPdk1−/−LacZ and POMCPdk1−/+LacZ mice. Immunohistochemistry using anti-β-galactosidase antibody revealed that numbers of POMC neurons of POMCPdk1−/−LacZ mice are similar to those of POMCPdk1−/+LacZ mice at the ages of 8, 16, and 24 wk (Fig. 2, A and B, and Supplemental Fig. 2, a and b). To investigate the effect of loss of PDK-1 in POMC neurons on their function, we measured [Ca2+]i in single POMC neurons by use of ratiometric fura 2 microfluorometry combined with digital imaging followed by immunocytochemical staining. [Ca2+]i is considered a good indicator of the activity of neurosecretory cells (21, 32, 37, 41). In POMC neurons of control mice, leptin at 10−10 M induces increases in [Ca2+]i (Fig. 2C). However, in POMC neurons of POMCPdk1−/− mice, leptin at the same concentration cannot induce Ca2+ influx (Fig. 2D). Although among 15 POMC neurons of control mice seven (46.6%) responded to leptin, none of eight POMC neurons responded to leptin (Fig. 2F). Furthermore, we examined the effect of PI(3)K inhibitor LY-294002 at 50 μM. LY-294002 treatment also abolished leptin-induced Ca2+ influx (Fig. 2E). Only one neuron (6.6%) among 15 POMC neurons of POMCPdk1−/− mice responded to leptin (Fig. 2F). These data indicate that leptin-stimulated Ca2+ influx in POMC neurons is PDK-1-dependent and suggest that loss of PDK-1 may blunt responses to leptin in POMC neurons.

**Effects of loss of PDK1 on expression of neuropeptides.** To investigate effects of loss of PDK-1 in POMC neurons on expression levels of neuropeptides in hypothalamus, we performed real-time PCR of Pomc, AgRP, and Npy genes. Real-time PCR demonstrated that Pomc gene expression in hypothalamus of POMCPdk1−/− mice decreased by ~50% compared with control mice (Fig. 3A). Immunohistochemistry using anti-POMC antibody also indicated that POMC protein expression decreased compared with control mice (Fig. 3B). We examined subcellular localization of FoxO1 in hypothalamus of POMCPdk1−/− and POMCPdk1+/− mice. Immunohistochemistry using anti-FoxO1 demonstrated that FoxO1 is localized in nucleus in POMCPdk1−/− mice (Fig. 3C). The ChIP assay indicated that endogenous FoxO1 in hypothalamus of POMCPdk1−/− mice bound to and phosphorylated STAT3 were dissociated from Pomc promoter (Fig. 3D). These data indicate that loss of PDK-1 in POMC neurons increases nuclear localization and binding to Pomc promoter of FoxO1 and decreases Pomc gene expression.

Selective apoptotic effects of loss of PDK1 on corticotrophs. Cre-recombinase is also expressed in corticotrophs in anterior pituitary (40). Serum corticosterone levels of POMCPdk1−/− mice were suppressed significantly at both fed and fasted states at the age of 16 wk (Fig. 4A). Furthermore, these mice showed fasting hypoglycemia and increased insulin sensitivity (Supplemental Fig. 3, a–d). Immunohistochemistry using anti-ACTH and anti-β-galactosidase antibodies indicated that corticotrophs in anterior pituitary from POMCPdk1−/− mice were decreased dramatically compared with control mice and the intermediate lobe of POMCPdk1−/− mice was extremely thin (Fig. 4B). In contrast, immunohistochemistry using anti-ACTH
antibody demonstrated that corticotrophs and melanotrophs in anterior and intermediate pituitary of POMCPdk1−/− mice at embryonic day 18.5 existed normally (Supplemental Fig. 4). These data suggest that loss of PDK-1 in corticotrophs does not affect development of corticotrophs but leads to loss of corticotrophs and melanotrophs postnatally. To investigate the mechanism of loss of corticotrophs and melanotrophs, we examined expression levels of p27 (Cdkn1b) and Bim in
The transcriptional block should be relieved by the Cre recombinase gene (CNFoxO1POMC), ubiquitous from the Rosa26R26floxneoCassette (33). The ES cell clones carrying the R26R26floxneoCNFoxO1, or R26R26floxneoΔ256FoxO1-targeted alleles were identified, and chimeras were generated. Mice heterozygous for R26R26floxneoCNFoxO1 or R26R26floxneoΔ256FoxO1 appeared normal and were fertile. To generate mice overexpressing POMC neuron-specific mutant FoxO1, R26R26floxneoCNFoxO1 or R26R26floxneoΔ256FoxO1 heterozygotes were bred with POMCCre mice (2) to generate R26R26floxneoCNFoxO1POMCCre (CNFoxO1POMC) or R26R26floxneoΔ256FoxO1POMCCre (Δ256FoxO1POMC) double heterozygotes, hereafter designated mutants, which were obtained at the predicted Mendelian ratio (~25%).

CNFoxO1POMC mice show mild obesity and increased food intake. To determine whether FLAG-tagged mutant FoxO1 is expressed in the POMC neurons of CNFoxO1POMC mice, we crossed these mice with CAG-CAT-LacZ mice (29) to generate CNFoxO1POMC/LacZ triple heterozygotes (Supplemental Fig. 6). Immunofluorescence demonstrated that CNFoxO1 was expressed in the POMC neurons (Fig. 5A). Approximately 90% of cells stained for β-galactosidase also stained with FLAG (Fig. 5B). We also analyzed whether the expression of overall hypothalamic FoxO1 was increased in CNFoxO1POMC mice by using primers that recognize both endogenous and transgenic FoxO1 mRNA. Hypothalamic FoxO1 expression was 60–70% higher in CNFoxO1POMC mice compared with controls (Fig. 5C). Both male and female CNFoxO1POMC mice were found to have normal body weight until ~8–10 wk of age. However, after 12 wk of age, both male and female mutant mice tended to have increased body weight compared with control mice, although we detected statistically significant differences only in female mutant mice (Fig. 1B). Mutant mice did not show any significant differences in nasso-anal length (NAL) compared with control mice (data not shown). A CT scan revealed that the adipose tissue mass of mutant mice was similar to that of control mice, although female mutant mice tended to have increased adiposity (Supplementary Fig. 7). Serum leptin levels in mutant mice were similar to those in controls (data not shown). Although the food intake of male mutant mice was similar to that of control mice, the food intake of female mice was increased significantly over the course of 1 wk (Fig. 5D). Indirect calorimetric analysis found that the oxygen consumption and respiratory quotient of mutants were similar to those in control mice (data not shown). Locomotive activity of CNFoxO1POMC mice was similar to that of control mice (data not shown). These data suggest that overexpression of CNFoxO1 in POMC neurons increases food intake. Measurements of fed blood glucose, insulin levels, intraperitoneal glucose, and insulin tolerance tests revealed no significant differences between control and mutant mice (data not shown). In contrast, we could not detect any hypothalamic phenotype in Δ256FoxO1POMC mice (data not shown). We ana-
lyzed the expression of the transgene in pituitary gland as well as in the case of POMCPdk1−/− mice. Immunofluorescence to detect FLAG demonstrated that FLAG-CNFoxO1 was expressed in the anterior and intermediate lobes of the pituitary glands (Supplemental Fig. 8a). However, the serum corticosterone levels in the mutants were similar to those in control mice in both the fasting and fed states (Supplemental Fig. 8b).

To investigate the chronic effects of overexpression of the CNFoxO1 in POMC neurons on the expression of neuropeptide genes, we performed real-time PCR using total RNA from the hypothalamic area. The real-time PCR analysis demonstrated that overexpression of CNFoxO1 in POMC neurons decreased the expression levels of Pomc and Npy genes during the fed state (Fig. 5E).

A ChIP assay using hypothalamic samples from the fed state demonstrated that exogenous FLAG-CNFoxO1 interacted with the Pomc promoter and that phosphorylated STAT3 was dissociated from the Pomc promoter region (Fig. 5F). These data suggest that CNFoxO1 interacts with the Pomc promoter and inhibits its gene expression.
FoxO1 in POMC neurons regulates Pmc gene expression. To confirm that FoxO1 works downstream of PDK-1 in POMC neurons in vivo, we crossed \( CN_{FoxO1}^{POMC} \) or \( H256_{FoxO1}^{POMC} \) with \( Pdk1_{fluox}^{/-} \) mice to generate \( CN_{FoxO1}^{POMC}Pdk1^{/-} \) or \( H256_{FoxO1}^{POMC}Pdk1^{/-} \) mice. Then, we crossed them with \( Pdk1_{fluox}^{/-} \) to generate \( CN_{FoxO1}^{POMC}Pdk1^{/-} \) mice (Supplemental Fig. 9). If FoxO1 mediates most actions of PDK-1 in POMC neurons, overexpression of \( H256_{FoxO1}^{POMC} \) may rescue phenotypes of \( POMCPdk1^{/-} \) mice.

Although body weights of both \( \Delta 256FoxO1^{POMC}Pdk1^{/-} \) and \( CN_{FoxO1}^{POMC}Pdk1^{/-} \) mice tended to be heavier than \( POMCPdk1^{/-} \) mice, body weight of only the \( CN_{FoxO1}^{POMC}Pdk1^{/-} \) mice increased significantly after 16 wk of age compared with \( POMCPdk1^{/-} \) mice (Fig. 6A). Total daily food intake of \( CN_{FoxO1}^{POMC}Pdk1^{/-} \) mice increased significantly compared with...
Fig. 4. Knockout of Pdk1 in anterior pituitary causes hypocorticosteronemia. A: serum corticosterone levels of control (gray bar) and POMCPdk1−/− (blue bar) mice during fasting and random-fed states at 16 wk. Serum corticosterone levels were measured as described in MATERIALS AND METHODS. Data represent means ± SE of 20 mice/genotype. *Statistically significant difference between control and POMCPdk1−/− mice (*P < 0.001 by one-factor ANOVA). B: representative immunofluorescence of ACTH (left) and β-galactosidase (middle) in pituitary of POMCPdk1−/−LacZ (top) and POMCPdk1−/−LacZ mice (bottom). Green and red indicate ACTH and β-galactosidase, respectively. Scale bars, 50 μm. C: expression of p27 and Bim in pituitaries of POMCPdk1−/− (gray bar) and POMCPdk1−/− mice (blue bar). Data were corrected for expression level of β-actin and represent means ± SE of 6 mice/genotype. *Statistically significant difference (*P < 0.005 by one-factor ANOVA). D: representative immunofluorescence of cleaved caspase 3 in pituitary of POMCPdk1−/− mice. Green and blue indicate cleaved caspase 3 and DAPI, respectively. White arrows, cells stained with anti-cleaved caspase 3 antibody. E: expression of Cre in hypothalamic regions (left) and pituitaries (right) of POMCPdk1−/− (gray bar) and POMCPdk1−/− mice (blue bar). Data were corrected for expression level of β-actin and represent means ± SE of 6 mice/genotype. *Statistically significant difference (*P < 0.001 by one-factor ANOVA).

POMCPdk1−/− and Δ256FoxO1POMCPdk1−/− mice (Fig. 6B). Real-time PCR analysis demonstrated that Pome gene expression decreased by ~50% and Agrp gene expression of CNFoxO1POMCPdk1−/− mice increased by ~80% compared with POMCPdk1−/− and Δ256FoxO1POMCPdk1−/− mice (Fig. 6C). These data suggest that ectopic expression of CNFoxO1 has additive effects on inhibition of Pome gene expression and increased food intake induced by loss of PDK-1 in POMC neurons. Furthermore, a ChIP assay using hypothalamic samples from the fed Δ256FoxO1POMCPdk1−/− mice demonstrated that exogenous FLAG-Δ256FoxO1 interacted with the Pome promoter but endogenous FoxO1 did not bind to Pome promoter (Fig. 6D, lane 4), although endogenous FoxO1 interacted with Pome promoter in POMCPdk1−/− mice (Fig. 6D, lane 3). Interestingly, phosphorylated STAT3 in Δ256FoxO1POMCPdk1−/− mice did not interact with Pome promoter (Fig. 6D, lane 4) as well as in POMCPdk1−/− and CNFoxO1POMCPdk1−/− mice (Fig. 6D, lanes 3 and 5, respectively). These data suggest that both CN and Δ256FoxO1 can bind to Pome promoter and interfere with the interaction between phosphorylated STAT3 and Pome promoter.
DISCUSSION

In the present study, we demonstrated that both male and female POMCpdk1−/− mice exhibit progressive body weight gain and increased food intake, although these mice also have hypogonadism due to loss of corticotrophs. Belgardt et al. (4) reported that PDK-1 deficiency in POMC neurons caused similar phenotypes, including hyperphagia, increased body weight, and impaired glucose metabolism, caused by reduced hypothalamic Pomc expression in young mice, but thereafter, PDK-1-deficient mice exhibited progressive reduction of body weight, food intake, serum leptin concentration, and epigonal fat pad mass by progressive, severe hypogonadism due to loss of POMC-expressing corticotrophs in the pituitary. From their and our studies, PDK-1 in POMC neurons plays an important role for regulation of food intake. Indeed, in the present study, we demonstrated that [Ca2+]i in single POMC neuron in response to leptin was blunted in POMCpdk1−/− mice. These data suggest that PDK-1 in POMC neurons is important for some actions of leptin.

However, some data of the present study are inconsistent with the previous reports. For example, Belgardt et al. reported that POMCpdk1−/− mice show hyperleptinemia, but we did not detect hyperleptinemia in POMCpdk1−/− mice despite the presence of leptin resistance. Furthermore, Belgardt et al. reported that a truncated Δ256FoxO1 rescued hypothalamic phenotypes of POMCpdk1−/− mice, but we could not. We speculate that these differences resulted from experimental methods. One of them may be due to different genetic background that the mice had. Belgardt et al. performed a backcross of the mice they used onto a C57BL/6 background (4); in contrast, we used mixed-background mice. Another is the difference of targeting vector of Rosa26 locus (33). Belgardt et al. generated a Rosa26 locus targeting vector using CAGS promoter-driven expression of the Δ256FoxO1 mutant, but we just knocked in the mutant FoxO1 in Rosa26 locus. The different construct of targeting vectors may have affected the expression level of mutant FoxO1. Furthermore, whereas we used a FLAG-tagged version of Δ256FoxO1, Belgardt et al. used an untagged version. Therefore, both Δ256FoxO1 mutants may differ in their actions on the Pomc promoter to compete with Stat3 binding and/or cofactors.

Several studies about genetically modified mice in a POMC neuron-specific manner have suggested roles of insulin signaling molecules in POMC neurons. However, POMC-restricted insulin receptor (Ir) knockout mice (IrΔPOMC) did not exhibit any altered energy homeostasis (16). Furthermore, POMC neuron-specific Irs2 knockout mice (POMCcreIrs2KO) did not show hyperphagia, obesity, and increased body length, which suggested no alteration of melanocortin action (8). These studies suggested that both Ir and Irs2 in POMC neurons did not mediate insulin action on regulation of food intake and body weight. However, PDK-1 is a convergent molecule of both insulin and leptin signaling. Therefore, even if insulin signaling was defective, PDK-1 activation occurred by activation of leptin signaling. Alternatively, a receptor other than Ir, for example insulin-like growth factor-1 receptor (Igf1r), in POMC neurons can compensate for inactivation through Ir (35). Similarly, insulin receptor substrates (Irs) other than Irs2 in POMC neurons can compensate for Irs2 action (7, 39).
Nuclear localization of FoxO1 in POMC neurons of *POMCPdk1<sup>−/−</sup>* mice increased. Furthermore, the ChIP assay demonstrated that FoxO1 bound to *Pomc* promoter increased in *POMCPdk1<sup>−/−</sup>* mice. These events lead to decreased expression of *Pomc*. Furthermore, the selective overexpression of CNFoxO1 in POMC neurons leads to the inhibition of *Pomc* expression. Interestingly, body weight of *CNFoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup>* mice, which lack PDK-1 and also overexpress CNFoxO1 in POMC neurons, was more obese than that of *POMCPdk1<sup>−/−</sup>* mice. These data define the role of FoxO1 in POMC neurons as an inhibitor of *Pomc* expression in vivo. The present study supports the previous reports in which CNFoxO1 expressed in the hypothalamic ARC by stereotactic adenoviral delivery inhibited *Pomc* gene expression (13) and confirms that FoxO1 in POMC neurons regulates *Pomc* gene expression. During the revision of this manuscript, Plum et al. (26) reported that FoxO1 ablation in POMC neurons increased α-melanocyte-stimulating hormone (α-MSH) due to increased expression of carboxypeptidase E and decreased food intake. We could not exclude the possibility that FoxO1 affected α-MSH protein level directly. From these findings, FoxO1 in POMC neurons increases food intake.

We also used another mutant FoxO1 (*Δ256FoxO1<sup>POMC</sup>*) transgenic mice (*Δ256FoxO1<sup>POMC</sup>*), in which a truncated FoxO1 that retains its NH<sub>2</sub> terminus and DNA binding domain but lacks the carboxyl terminal transactivation domain was selectively expressed in POMC neurons and examined whether overexpression of *Δ256FoxO1* in POMC neurons could rescue phenotypes of *POMCPdk1<sup>−/−</sup>* mice or not. However, *Pomc* expression level in *Δ256FoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup>* was similar to that in *POMCPdk1<sup>−/−</sup>* mice, and the hypothalamic phenotype of *POMCPdk1<sup>−/−</sup>* mice was not rescued. We have to remember that this transactivation-defective FoxO1 is not sufficient for complete loss-of-function of FoxO1 and that *Δ256FoxO1* used in the present study still has the NH<sub>2</sub> terminus and forkhead DNA-binding domain, and these domains could bind to some cofactors for transcription of FoxO1 target genes in a tissue- or cell type-specific manner (24). Interestingly, the ChIP assay demonstrated that *Δ256FoxO1* bound to *Pomc* promoter and phosphorylated STAT3 dissociated from *Pomc* promoter in *Δ256FoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup>* mice as well as *POMCPdk1<sup>−/−</sup>* and *CNFoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup>* mice. These data support our finding that *Δ256FoxO1* still inhibits STAT3 binding to *Pomc* promoter and the NH<sub>2</sub> terminus of FoxO1 may play an important role for inhibition of STAT3 binding. However, we could not see any phenotypes of *Δ256FoxO1<sup>POMC</sup>* mice. *Δ256FoxO1* still has two intact Akt phosphorylation sites, Thr<sub>24</sub> and Ser<sub>253</sub> (22). It has been reported that phosphorylation of the first and second Akt phosphorylation sites can affect DNA binding and regulate transcriptional activity of FoxOs (5, 36, 38, 42). Therefore, loss of PDK-1 may enhance DNA binding of *Δ256FoxO1*, and *Δ256FoxO1* can inhibit STAT3 binding to *Pomc* promoter. However, in the presence of intact PDK-1, *Δ256FoxO1* may be phosphorylated in the nucleus and cannot bind to *Pomc* promoter and lead to no phenotype of *Δ256FoxO1<sup>POMC</sup>* mice. Furthermore, it is suggested that the carboxyl terminus of FoxO1 may have an additive effect on inhibition of *Pomc* gene expression other than an inhibition of STAT3 binding to *Pomc* promoter by the NH<sub>2</sub> terminus of *Δ256FoxO1*.

The *Pomc* promoter is also active in corticotrophs (40). We have demonstrated that knockout of *Pdk1* in corticotrophs and melanotrophs lead to apoptosis but not in POMC neurons in hypothalamus. It is impossible to think that knockout of *Pdk1* in corticotrophs and melanotrophs affects pituitary development because the histological structure and ACTH expression level in pituitary of *POMCPdk1<sup>−/−</sup>* mice at embryonic day 18.5 are similar to those in control mice. These data suggest that PDK-1 has different roles in a tissue-specific manner and are consistent with the previously described studies about other
tissue-specific Pdk1 knockout mice, such as liver-specific (25) and pancreatic β-cell-specific Pdk1 knockout mice (11).

The present study confirmed that PDK-1 in POMC neurons plays an important role for regulation of food intake and that PDK-1/FOXO1 in POMC neurons plays an important role for regulation of food intake and FoxO expression. These results are an important step in establishing the mechanism by which PDK-1/FOXO1 in POMC neurons regulates energy homeostasis.

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DISCLOSURES

No conflicts of interest are reported by the author(s).

REFERENCES


