POMCPdk1 pathway in POMC neurons regulates Pomc expression and food intake

Kristy Iskandar,1* Yongheng Cao,1,2,9* Yoshitake Hayashi,1 Masanori Nakata,3 Eisuke Takano,3 Toshihiko Yada,3 Changliang Zhang,4 Wataru Ogawa,5 Miyo Oki,2 Streamson Chua, Jr.,6 Hiroshi Itoh,7 Tetsuo Noda,8 Masato Kasuga,5,9 and Jun Nakae2,7

1Division of Molecular Medicine and Medical Genetics, International Center for Medical Research and Treatment; 221st Century Center of Excellence Program for Signal Transduction Disease: Diabetes Mellitus as Model, Department of Internal Medicine, Division of Diabetes, Metabolism, and Endocrinology, Kobe University Graduate school of Medicine, Kobe; 3Division of Integrative Physiology, Department of Physiology, Jichi Medical University, School of Medicine, Tochigi; 4Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan; 5Division of Diabetes, Metabolism, and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan; 6Departments of Medicine and Neuroscience, Albert Einstein College of Medicine, Bronx, New York; 7Frontier Medicine on Metabolic Syndrome, Division of Endocrinology, Metabolism and Nephrology, Department of Internal Medicine, Keio University School of Medicine; 8Department of Cell Biology, Japanese Foundation for Cancer Research, Cancer Institute; 9Research Institute, International Medical Center of Japan, Tokyo, Japan

Submitted 20 August 2009; accepted in final form 20 January 2010

Iskandar K, Cao Y, Hayashi Y, Nakata M, Takano E, Yada T, Zhang C, Ogawa W, Oki M, Chua S Jr, Itoh H, Noda T, Kasuga M, Nakae J. PDK-1/FoxO1 pathway in POMC neurons regulates POMC expression and food intake. Am J Physiol Endocrinol Metab 298: E787–E798, 2010. First published January 26, 2010; doi:10.1152/ajpendo.00512.2009.—Both insulin and leptin signaling converge on phosphatidylinositol 3-0H kinase [PI(3)K]/3-phosphoinositide-dependent protein kinase-1 (PDK-1)/protein kinase B (PKB, also known as Akt) in proopiomelanocortin (POMC) neurons. Forkhead box-containing protein-O1 (FoxO1) is inactivated in a PI(3)K-dependent manner. However, the interrelationship between PI(3)K/PDK-1/Akt and FoxO1, and the chronic effects of the overexpression of FoxO1 in POMC neurons on energy homeostasis has not been elucidated. To determine the extent to which PDK-1 and FoxO1 signaling in POMC neurons was responsible for energy homeostasis, we generated POMC neuron-specific Pdk1 knock-out mice (POMCPdk1–/–) and mice selectively expressing a constitutively nuclear (CN)FoxO1 or transactivation-defective (Δ256)FoxO1 in POMC neurons (CNFoxO1POMC or Δ256FoxO1POMC). POMCPdk1–/– mice showed increased food intake and body weight accompanied by decreased expression of POMC gene. The CNFoxO1POMC mice exhibited mild obesity and hyperphagia compared with POMCPdk1–/– mice. Although expression of the CNFoxO1 made POMCPdk1–/– mice more obese due to excessive suppression of POMC expression, overexpression of Δ256FoxO1 in POMC neurons had no effects on metabolic phenotypes and POMC expression levels of POMCPdk1–/– mice. These data suggest a requirement for PDK-1 and FoxO1 in transcriptional regulation of POMC and food intake.

phosphoinositide-dependent protein kinase-1; forkhead box-containing protein O1; proopiomelanocortin neurons; POMC; food intake

OBESITY IS THE RESULT OF AN IMBALANCE BETWEEN ENERGY INTAKE AND EXPENDITURE. Energy intake is determined mainly by food intake. Recent findings about the regulation of food intake have suggested that two populations in the arcuate nucleus (ARC) of the hypothalamus, including agouti-related protein (AgRP)/neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons, have important roles in energy homeostasis (3, 9, 19, 31).

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, which activates transcription 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 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 increases 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 transactiva-
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 Pkgneo 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-Pkgneo-loxP-CN (or Δ256) FoxO1-bpA, was inserted into the PovC/Ascl sites of the pR26-1 to generate a targeting vector. A diphertheria toxin expression cassette (DT) was present within pR26-1 for negative selection. The targeting vector was electroporated into embronic 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 R26flxne-CN (or Δ256) FoxO1 allele to their progeny. The R26flxne-CN (or Δ256) FoxO1 allele was examined on a B6129 mixed genetic background.

Breeding colonies were maintained by mating R26flxne-CNfoxO1 or R26flxneΔ256foxO1 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 R26Δ256foxO1-F and R26Δ256foxO1-R mice were 5′-ATGGACTCAAAAGACGATGAC-3′ (sense) and 5′-GCTTTCCTCATTAAGAGGTCT-3′ (antisense) and 5′-TGGAGGCCATCAGACTTAGAC-3′ (antisense); Npy 5′-AAAGCGGCAAGTGGCCCGAGG-3′ (sense) and 5′-GTTCITCCTATTAGAGTCT-3′ (antisense); Cart 5′-ATCTACTCTGTCGTGATGAT-3′ (sense) and 5′-TTCTCCTACGCTGCTGT-3′ (antisense). Endogenous FoxO1 was amplified using FoxO1-S969 5′-GATAAGGCGACCAGCAACAGG-3′ (sense) and FoxO1-A1414 5′-TGGCCAGCAGGGTGATAC-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 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 fluor or R 555 goat anti-mouse IgG (H + L) (A21424), Molecular Probes] to double stain for FLAG and β-galactosidase.

**Chromatin immunoprecipitation assay.** Hypothalamus samples were trypsinized with frequent pipetting and fixed with 1% parafor-
maldehyde (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 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 fluor or R 555 goat anti-mouse IgG (H + L) (A21424), Molecular Probes] to double stain for FLAG and β-galactosidase.

**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, Shintechno). 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 Pomc 5′-AGACCTCTATAGTGGTGGGGA-3′ (sense) and 5′-AGCGGATTGCCATGACGCT-3′ (antisense); AgRP 5′-ATGCTGACAGGAATTTGGCAGT-3′ (sense) and 5′-TGAGGGCCATCAGACTTAGAC-3′ (antisense); Npy 5′-AAAGCGGCAAGTGGCCCGAGG-3′ (sense) and 5′-GTTCITCCTATTAGAGTCT-3′ (antisense); Cart 5′-ATCTACTCTGTCGTGATGAT-3′ (sense) and 5′-TTCTCCTACGCTGCTGT-3′ (antisense). Endogenous FoxO1 was amplified using FoxO1-S969 5′-GATAAGGCGACCAGCAACAGG-3′ (sense) and FoxO1-A1414 5′-TGGCCAGCAGGGTGATAC-3′ (antisense).

**Analytic procedures and tolerance tests.** We carried out all assays in duplicate; each value represents the means of two independent determinations. Intrapерitoneal 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).
min. After trituration, the cell suspension was centrifuged at 100 g for 5 min. The pellet was resuspended in HKRB and distributed onto coverslips.

**Measurements of [Ca\(^{2+}\)]\(_i\), in single arc neurons.** Briefly, after incubation with 2 μM flura 2-AM for 30 min at room temperature, the cells were mounted in a chamber and superfused with HKRB containing 10 mM glucose at 1 ml/min at 33°C. Cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was measured by ratiometric flura 2 fluorescence imaging with intensified charge-coupled device camera, and the ratio image was produced by an Aquacosmos system (Hamamatsu Photonics). After [Ca\(^{2+}\)] measures, the cells were fixed with 4% paraformaldehyde for 30 min. Immunocytochemical identification of POMC neurons was performed using rabbit anti-POMC antibody (34) and ABC method (20). For treatment with LY-294002 (SABiosciences), cells were treated with LY-294002 at 50 μM for 30 min and stimulated with leptin and glutamate.

**Statistics.** We calculated descriptive statistics using analysis of variance followed by Fisher’s test (Statview, SAS Institute). P values < 0.05 were considered significant.

**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 Pdk1\(^{floxed}\) mice (12) with POMCCre transgenic mice (2) and then crossed the resulting Pdk1\(^{floxed}\)/POMCCre offspring (POMCPdk1\(^{-/-}\)) with Pdk1\(^{floxed}\)/POMCCre (POMCPdk1\(^{-/-}\)) mice. The genotypes of animals born from the latter cross conform to the expected ratio (data not shown). To confirm whether PDK-1 expression levels in POMC neuron 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 Pdk1\(^{floxed}\)/POMCCre 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 do not express 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 quotient 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 Ca\(^{2+}\) 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 [Ca\(^{2+}\)]\(_i\) in single POMC neurons by use of ratiometric flura 2 microfluorometry combined with digital imaging followed by immunocytochemical staining. [Ca\(^{2+}\)]\(_i\) 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 [Ca\(^{2+}\)]\(_i\) (Fig. 2C). However, in POMC neurons of POMCPdk1\(^{-/-}\) mice, leptin at the same concentration cannot induce Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 mainly in cytosol in POMCPdk1\(^{-/-}\) mice but that ~80% of 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
pituitary by real-time PCR. Although Cdkn1b expression was suppressed, Bin expression was increased significantly in pituitary of POMCPdk1+/−/− mice (Fig. 4C). Furthermore, cleaved caspase 3 was detected in pituitary of POMCPdk1+/−/− mice but not detected in control mice by immunohistochemistry (Fig. 4D and data not shown). In contrast, we could not detect any cleaved caspase 3-positive cells in hypothalamus of POMCPdk1+/−/− mice (data not shown). Furthermore, real-time PCR of Cre demonstrated that Cre expression in hypothalamus of POMCPdk1+/−/− was similar to that in POMCPdk1+/−/− mice but Cre expression in pituitary of POMCPdk1+/−/− was reduced significantly (Fig. 4E). These data suggest that loss of PDK-1 in corticotrophs and melanotrophs leads to apoptosis selectively but not in hypothalamic POMC neurons.

**Generation of POMC neuron-specific mutant FoxO1 transgenic mice.** PIP3-mediated activation of PKD-1 activates an enzyme cascade that includes Akt and members of the atypical PKC family. Among the targets of activated Akt are mTOR and the forkhead transcription factor FoxO1 (19). Recently, it was reported that FoxO1 in hypothalamus is involved in regulating neuropeptide expression and food intake (13, 14). Pome expression partially depends on the PDK-1-FoxO1 signaling pathway. It has already been reported that FoxO1 in POMC neurons inhibits Pome gene expression (4, 13).

To investigate whether activated FoxO1 mediates effects of knockout of Pdk1 in POMC neurons, we generated POMC neuron-specific mutant FoxO1 transgenic mice. A conditional genetic system was used to express CNFoxO1, in which three Akt phosphorylation sites were substituted for alanine (T24A/S253A/S316A: 3A) (23), or a transactivation-defective truncated Δ256FoxO1 (22), in which the carboxyl terminal transactivation domain (Supplemental Fig. 5a), in a Cre-dependent manner and potentially in any tissue. The FLAG-tagged mutant FoxO1, in which three Akt phosphorylation sites were substituted for alanine (T24A/S253A/S316A: 3A) (23), or a transactivation-defective truncated Δ256FoxO1 (22), in which the carboxyl terminal transactivation domain (Supplemental Fig. 5a), in a Cre-dependent manner and potentially in any tissue. The ubiquitously expressed Rosa26 locus was modified by gene targeting in ES cells (Supplemental Fig. 5b). The FLAG-tagged mutant mouse FoxO1 cDNA was placed 3′ to a floxed neomycin-resistant expression cassette, floxneo, which should block the transcription of the mutant FoxO1 from the Rosa26 promoter. This transcriptional block should be relieved by the Cre recombinase-mediated excision of the floxneo cassette (33). The ES cell clones carrying the R26loxneoCNFoxO1, or R26loxneoΔ256FoxO1-targeted alleles were identified, and chimeras were generated. mice heterozygous for R26loxneoCNFoxO1 or R26loxneoΔ256FoxO1 appeared normal and were fertile.

To generate mice overexpressing POMC neuron-specific mutant FoxO1, R26loxneoCNFoxO1 or R26loxneoΔ256FoxO1 heterozygotes were bred with POMCCre mice (2) to generate R26loxneoCNFoxO1/POMCCre (CNFoxO1/POMC) or R26loxneoΔ256FoxO1/POMCCre (Δ256FoxO1/POMC) double heterozygotes, hereafter designated mutants, which were obtained at the predicted Mendelian ratio (~25%).

**CNFoxO1/POMC mice show mild obesity and increased food intake.** To determine whether FLAG-tagged mutant FoxO1 is expressed in the POMC neurons of CNFoxO1/POMC mice, we crossed these mice with CAG-CAT-LacZ mice (29) to generate CNFoxO1/POMC/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 CNFoxO1/POMC mice by using primers that recognize both endogenous and transgenic FoxO1 mRNA. Hypothalamic FoxO1 expression was 60–70% higher in CNFoxO1/POMC mice compared with controls (Fig. 5C). Both male and female CNFoxO1/POMC 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). Male mutant mice did not show any significant differences in naso-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 CNFoxO1/POMC 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 Δ256FoxO1/POMC mice (data not shown). We ana-

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**Fig. 1.** Generation and metabolic characterization of POMCPdk1−/− mice. See text for definitions. A: representative double immunofluorescence of β-galactosidase and PDK-1 in hypothalamic regions of POMCPdk1−/− LacZ and POMCPdk1+/− LacZ mice in ad libitum-fed mice at 12 wk. Red, green, and blue indicate β-galactosidase (left), PDK-1 (middle), and DAPI, respectively. Scale bars, 50 μm. B: body weight of male (left) and female (right) control (□), CNFoxO1/POMC (●), and POMCPdk1−/− (○) mice. Data represent means ± SE of 35–40 mice/genotype. *Statistically significant differences between control and POMCPdk1−/− mice; **statistically significant differences between control and CNFoxO1/POMC mice (P < 0.001 and **P < 0.05 by one-factor ANOVA). C: adiposity of control (gray bar) and POMCPdk1−/− (blue bar) mice at 12 wk under normal chow diet. Skeletal muscle mass, adipose tissue (sum of visceral and subcutaneous fats), visceral fat, and subcutaneous fat masses were calculated as described in MATERIALS AND METHODS. Data are demonstrated as %body weight and represent means ± SE from 10 mice/genotype. Statistically significant differences between control and CNFoxO1/POMC mice (P < 0.001 and **P < 0.05 by one-factor ANOVA). D: male and female 4-day food intake of control (gray bar) and POMCPdk1−/− mice (blue bar) at 12 wk. Data represent means ± SE of 15 mice/genotype. *Statistically significant differences between control and POMCPdk1−/− mice (P < 0.001 and **P < 0.05 by one-factor ANOVA). E: F: average of VO2 (E) and respiratory quotient (RQ, F) of POMCPdk1−/− (gray bar) and POMCPdk1−/− mice (blue bar) under normal diet at 12 wk. Data represent means ± SE of 6 male mice/genotype. Measurements of VO2 were performed for 72 h, with the 1st day allowing mice to acclimate to the cage environment. G and H: effects of ip leptin injection on body weight and food intake at 12 wk. Leptin ip injection into control (C, n = 10) or POMCPdk1−/− mice (●, n = 5) was performed for 3 consecutive days, and body weight (G) and food intake (H) were monitored for 7 days. Data represent %body weight and food intake of mice injected with saline in each genotype. G: statistically significant differences between control and POMCPdk1−/− mice (P < 0.001 and **P < 0.05 by one-factor ANOVA).
lyzed the expression of the transgene in pituitary gland as well as in the case of POMCPdk1<sup>−/−</sup> mice. Immunofluorescence to detect FLAG demonstrated that FLAG-CNFoxO1 was expressed in the anterior and intermediate lobes of the pituitary glands (Supplemental Fig. 8a).

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).

Fig. 2. Loss of PDK-1 abolishes Ca<sup>2+</sup> influx in response to leptin in POMC neurons. A: representative immunofluorescence of β-galactosidase in hypothalamic regions of POMCPdk1<sup>+/+LacZ</sup> (left) and POMCPdk1<sup>−/−LacZ</sup> mice (right). Green and blue indicate β-galactosidase and DAPI, respectively. Scale bars, 50 μm. B: cell count of β-galactosidase-positive cells in ARC of POMCPdk1<sup>+/+LacZ</sup> (gray bar) and POMCPdk1<sup>−/−LacZ</sup> (blue bar) mice at 16 wk. C: oscillatory increase by leptin of [Ca<sup>2+</sup>]<sub>i</sub>, in the presence of 2.8 mM glucose was induced by leptin (10<sup>−10</sup> M) or glutamate (10<sup>−4</sup> M) in POMC neurons (n = 15) of POMCCre mice. D: leptin-induced increase of [Ca<sup>2+</sup>]<sub>i</sub>, in the presence of 2.8 mM glucose was blunted in POMC neurons (n = 8) of POMCPdk1<sup>−/−</sup> mice, while glutamate (10<sup>−4</sup> M) induced the oscillatory increase of [Ca<sup>2+</sup>]<sub>i</sub>. E: quantification of POMC neurons in which leptin (10<sup>−10</sup> M) or glutamate (10<sup>−4</sup> M) induced the oscillatory increase of [Ca<sup>2+</sup>]<sub>i</sub>. Data represent %response neurons among POMC neurons examined. Gray, blue, and magenta bars indicate %leptin-responsive neurons among POMC neurons of POMCCre and POMCPdk1<sup>−/−</sup> mice and POMC neurons treated with LY-294002, respectively.
FoxO1 in POMC neurons regulates Pomp gene expression. To confirm that FoxO1 works downstream of PDK-1 in POMC neurons in vivo, we crossed CNFoxO1POMC or Δ256FoxO1POMC with Pdk1flox/+ mice to generate CNFoxO1POMC/POMC Pdk1flox/+ (CNFoxO1POMC Pdk1flox/+ or Δ256FoxO1POMC POMCPdk1flox/+ (Δ256FoxO1POMCPdk1flox/+)) mice. Then, we crossed them with Pdk1flox/+ to generate CNFoxO1POMC Pdk1flox/+ or Δ256FoxO1POMC Pdk1flox/+ mice (Supplemental Fig. 9). If FoxO1 mediates most actions of PDK-1 in POMC neurons, overexpression of Δ256FoxO1 may rescue phenotypes of POMC Pdk1flox/+ mice.

Although body weights of both Δ256FoxO1POMC Pdk1flox/+ and CNFoxO1POMC Pdk1flox/+ mice tended to be heavier than POMC Pdk1flox/+ mice, body weight of only the CNFoxO1POMC Pdk1flox/+ mice increased significantly after 16 wk of age compared with POMC Pdk1flox/+ mice (Fig. 6A). Total daily food intake of CNFoxO1POMC Pdk1flox/+ 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<sup>−/−</sup> (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. Statistical difference between control and POMCPdk1<sup>−/−</sup> mice (*P < 0.001 by one-factor ANOVA). B: representative immunofluorescence of ACTH (left) and β-galactosidase (middle) in pituitary of POMCPdk1<sup>−/−</sup>LacZ (top) and POMCPdk1<sup>−/−</sup>LacZ mice (bottom). Green and red indicate ACTH and β-galactosidase, respectively. Scale bars, 50 μm. C: expression of p27<sup>+</sup> and Bim in pituitaries of POMCPdk1<sup>−/−</sup> (gray bar) and POMCPdk1<sup>−/−</sup> mice (blue bar). Data were corrected for expression level of β-actin and represent means ± SE of 6 mice/genotype. Statistical difference (*P < 0.005 by one-factor ANOVA). D: representative immunofluorescence of cleaved caspase 3 in pituitary of POMCPdk1<sup>−/−</sup> mice. Green and blue indicate cleaved caspase 3 and DAPI, respectively. White arrows, cells stained with anti-cleaved caspase 3. E: expression of Cre in hypothalamic regions (left) and pituitaries (right) of POMCPdk1<sup>−/−</sup> (gray bar) and POMCPdk1<sup>−/−</sup> mice (blue bar). Data were corrected for expression level of β-actin and represent means ± SE of 6 mice/genotype. Statistical difference (*P < 0.001 by one-factor ANOVA).

POMCPdk1<sup>−/−</sup> and Δ256FoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup> mice (Fig. 6B). Real-time PCR analysis demonstrated that Pome gene expression decreased by ~50% and Agrp gene expression of CNFoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup> mice increased by ~80% compared with POMCPdk1<sup>−/−</sup> and Δ256FoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup> 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 Δ256FoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup> mice demonstrated that exogenous FLAG-Δ256FoxO1 interacted with the Pome promotor but endogenous FoxO1 did not bind to Pome promoter (Fig. 6D, lane 4), although endogenous FoxO1 interacted with Pome promoter in POMCPdk1<sup>−/−</sup> mice (Fig. 6D, lane 3). Interestingly, phosphorylated STAT3 in Δ256FoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup> and CNFoxO1<sup>POMC</sup>Pdk1<sup>−/−</sup> 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.
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 hypocorticosteronemia 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 epididymal fat pad mass by progressive, severe hypocortisolism 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 (Igfl1), 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).
was performed using indicated antibodies as described in MATERIALS AND METHODS. The ChIP assay demonstrated that FoxO1 that bound to POMCPdk1 and POMCPdk1/−/− mice during random-fed state. Immunoprecipitation of hypothalamic neuropeptide genes of POMCPdk1 and POMCPdk1/−/− mice at fed state. Data were corrected for expression level 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 Δ256FoxO1POMCPdk1/−/− mice as well as POMCPdk1/−/− and POMCPdk1/−/− mice. These data support our finding that Δ256FoxO1 still inhibits STAT3 binding to POMC promoter and the NH2 terminus of FoxO1 may play an important role for inhibition of STAT3 binding. However, we could not see any phenotypes of Δ256FoxO1POMC mice. Δ256FoxO1 still has two intact Akt phosphorylation sites, Thr24 and Ser253 (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 Δ256FoxO1POMC mice. Furthermore, it is suggested that the carboxyl terminus of FoxO1 may have an additive effect on inhibition of POMC promoter expression other than an inhibition of STAT3 binding to POMC promoter by the NH2 terminus of Δ256FoxO1.

The POMC promoter is also active in corticotroph (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/−/− 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

Nuclear localization of FoxO1 in POMC neurons of POMCPdk1/−/− mice increased. Furthermore, the ChIP assay demonstrated that FoxO1 that bound to POMC promoter increased in POMCPdk1/−/− 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 CNFoxO1POMC Pdk1/−/− mice, which lack PDK-1 and also overexpress CNFoxO1 in POMC neurons, was more obese than that of POMCPdk1/−/− 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 adeno viral 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) transgenic mice (Δ256FoxO1POMC), in which a truncated FoxO1 that retains its NH2 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/−/− mice or not. However, Pomc expression level in Δ256FoxO1POMCPdk1/−/− was similar to that in POMCPdk1/−/− mice, and the hypothalamic phenotype of POMCPdk1/−/− 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 NH2 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 used in POMC promoter is also active in corticotroph (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/−/− 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

Fig. 6. Effects of mutant FoxO1s in POMC neurons on metabolic characterization and neuropeptide expression of POMCPdk1/−/− mice. A: body weight of male control (○), POMCPdk1/−/− (●), CNFoxO1POMCPdk1/−/− (▲), and Δ256FoxO1POMCPdk1/−/− (△) mice. Data represent means ± SE of 20 mice/genotype. *Statistically significant difference between CNFoxO1POMCPdk1/−/− and POMCPdk1/−/− mice (*P < 0.05 by one-factor ANOVA). B: male 4-day food intake of control, POMCPdk1/−/−, Δ256FoxO1POMCPdk1/−/−, and CNFoxO1POMCPdk1/−/− mice at 24 wk. Data represent means ± SE of 15 mice/genotype. Statistically significant differences between CNFoxO1POMCPdk1/−/− and POMCPdk1/−/− or Δ256FoxO1POMCPdk1/−/− mice: *P < 0.005, **P < 0.01 by one-factor ANOVA. C: expression of hypothalamic neuropeptide genes of POMCPdk1/−/− (white bar), CNFoxO1POMCPdk1/−/− (black bar), and Δ256FoxO1POMCPdk1/−/− mice (gray bar) at fed state. Data were corrected for expression level of β-actin and represent means ± SE of 6 mice/genotype. *Statistically significant difference between CNFoxO1POMCPdk1/−/− and POMCPdk1/−/− or Δ256FoxO1POMCPdk1/−/− mice (*P < 0.005 by one-factor ANOVA). D: ChIP assay for Pomc promoter in control (lanes 1 and 2), POMCPdk1/−/− (lane 3), and Δ256FoxO1POMCPdk1/−/− (lane 4), and CNFoxO1POMCPdk1/−/− (lane 5) mice during random-fed state. Immunoprecipitation was performed using indicated antibodies as described in MATERIALS AND METHODS.
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 has 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.

ACKNOWLEDGMENTS

We thank Dr. Jun-ichi Miyazaki (Division of Stem Cell Regulation Research, G6, Osaka University Graduate School of Medicine) for providing us the CAG-CAT-Z transgenic mice, Dr. Thomas Ludwig (Department of Genetics and Development, Columbia University) for providing us the pR26-1 plasmid, and Dr. Susumu Seino (Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine) for kindly making his laboratory available to accomplish this work.

GRANTS

This work was supported by a grant for the 21st Century COE Program “Center of Excellence for Signal Transduction Disease: Diabetes Mellitus as a Model” from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M. Kasuga, a Grant-in-Aid for Creative Scientific Research from MEXT to M. Kasuga, a grant from Nippon Boehringer Ingelheim, to H. Hisadome, a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Japan) for the Promotion of Science to J. Nakae, a grant from Novo Nordisk Pharma to J. Nakae, and a grant from the NOVARTIS Foundation (Japan) for the Promotion of Science to J. Nakae.

DISCLOSURES

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

REFERENCES


