THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 48, pp. 37884–37894, November 26, 2010

Regulation of Ghrelin Signaling by a Leptin-induced Gene, Negative Regulatory Element-binding Protein, in the Hypothalamic Neurons*[S]

Received for publication, May 27, 2010, and in revised form, September 17, 2010 Published, JBC Papers in Press, September 28, 2010, DOI 10.1074/jbc.M110.148973

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Leptin, the product of the *ob* gene, plays important roles in the regulation of food intake and body weight through its receptor in the hypothalamus. To identify novel transcripts induced by leptin, we performed cDNA subtraction based on selective suppression of the polymerase chain reaction by using mRNA prepared from the forebrain of leptin-injected *ob/ob* mice. One of the genes isolated was a mouse homolog of human negative regulatory element-binding protein (NREBP). Its expression was markedly increased by leptin in the growth hormone secretagogue-receptor (GHS-R)-positive neurons of the arcuate nucleus and ventromedial hypothalamic nucleus. The promoter region of GHS-R contains one NREBP binding sequence, suggesting that NREBP regulates GHS-R transcription. Luciferase reporter assays showed that NREBP repressed GHS-R promoter activity in a hypothalamic neuronal cell line, GT1-7, and its repressive activity was abolished by the replacement of negative regulatory element in GHS-R promoter. Overexpression of NREBP reduced the protein expression of endogenous GHS-R without affecting the expression of ob-Rb in GT1-7 cells. To determine the functional importance of NREBP in the hypothalamus, we assessed the effects of NREBP on ghrelin action. Although phosphorylation of AMP-activated protein kinase α (AMPKα) was induced by ghrelin in GT1-7 cells, NREBP repressed ghrelin-induced AMPKα phosphorylation. These results suggest that leptin-induced NREBP is an important regulator of GHS-R expression in the hypothalamus and provides a novel molecular link between leptin and ghrelin signaling.

Obesity develops when food intake exceeds compensatory increases in energy expenditure, and energy is accumulated as fat (1). Food intake is controlled by the precise coordination between the neural circuitry and peripheral factors that derive from fat, gut, and pancreas. The peripheral factors transduce their signals by binding to the receptors in the hypothalamus and regulate production of the orexigenic and the anorexigenic peptides in the specific subsets of hypothalamic neurons (2). Thus, the hypothalamus is critical in integrating signals from the peripheral factors in the neural circuitry.

Leptin, an adipocyte-derived hormone (3), is a key negative regulator of food intake and energy expenditure. Circulating leptin enters the brain through the blood-brain barrier (4) and exerts its effects through binding to the long form of the leptin receptor ob-Rb (5) expressed in the hypothalamus, including the arcuate nucleus, ventromedial hypothalamic nucleus (VMH),[2] dorsomedial hypothalamic nucleus, lateral hypothalamic nucleus, and paraventricular hypothalamic nucleus (6). For example, in the hypothalamic arcuate nucleus, leptin suppresses food intake via decreased expression of the orexigenic peptides, neuropeptide Y, and agouti-related peptide and increased expression of the anorexigenic peptides, proopiomelanocortin, and cocaine- and amphetamine-regulated transcript (6–9).

Although the administration of leptin reverses obesity caused by its deficiency in mice and humans (10, 11), obesity caused by total deficiency of leptin is uncommon in humans. Instead, most obese humans are characterized by resistance to leptin. Some mechanisms are thought to be involved in the development of leptin resistance: circulating factors that bind to leptin and inhibit its physiological functions, such as C-reactive protein and the soluble form of the leptin receptor (12, 13), defects in the transport of leptin across the blood-brain barrier, and impairments of the intracellular signaling cascade from leptin receptor.

In the hypothalamus, binding of leptin to ob-Rb can mediate transcription of target genes mainly via the activation of the JAK2 signal transducer and activator of transcription 3 pathway

* This work was supported by Grant-in-aid for Scientific Research 17019047 on Priority Areas "Applied Genomics" from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported by Grant-in-aid for Scientific Research 13204074 on Priority Areas "Medical Genome Science" from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a grant provided by the Ichiro Kanehara Foundation; and a research grant on Priority Areas from Wakayama Medical University.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB546195.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: VMH, ventromedial hypothalamic nucleus; AMPK, AMP-activated protein kinase; GHS-R, growth hormone secretagogue-receptor; NREBP, negative regulatory element-binding protein.

(14). Among them, SOCS3 (suppressor of cytokine signaling 3) negatively regulates hypothalamic leptin signaling via the suppression of JAK2 activation and contributes to the development of a leptin-resistant state (15). Previously, White *et al.* (2000) have identified four novel leptin-induced transcripts (LRG-47, T cell-specific guanine nucleotide triphosphate-binding protein, RC10-11, and Stra-13) from a hypothalamic neuronal cell line, GT1-7, stimulated with leptin (16). However, the roles of leptin-induced molecules in feeding behavior and energy metabolism remain unknown except for neuropeptides and SOCS3. Therefore, the identification of leptin-induced transcripts is of substantial biomedical importance. In the present study, we identified a mouse homolog of human negative regulatory element-binding protein (NREBP), a transcriptional repressor, as a leptin-induced transcript, which repressed the expression of growth hormone secretagogue-receptor (GHS-R) in the hypothalamus.

EXPERIMENTAL PROCEDURES

Animals—Male C57BL/6J lean and *ob/ob* mice (8 to 10 weeks old) were obtained from our breeding colony using heterozygous (*ob*/+) breeding pairs. Mice were housed in specific pathogen-free facilities, in light (12 h light/dark cycle), temperature (22–25 °C), and humidity (50–60% relative humidity) controlled conditions. Mice were fed a standard diet (MF; Oriental Yeast, Tokyo, Japan) and water *ad libitum*. At all times, the experiments were performed under the control of the Animal Research Control Committee in accordance with the Guidelines for Animal Experiments of Wakayama Medical University and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

Injection of Leptin in ob/ob Mice—Mice who had fasted for 9 h (starting from 9:00 a.m.) were injected intravenously with PBS, pH 7.4, or recombinant mouse leptin (R&D Systems, Minneapolis, MN) dissolved with PBS at a dose of 10 μg/g body weight.

Cloning of Leptin-induced Sequences—Total RNAs from the forebrain of *ob/ob* mice 1 h after the intravenous injection of PBS or mouse leptin were prepared using TRI reagent (Molecular Research Center, Cincinnati, OH) as described previously (17). Isolated mRNA from 200 μg of total RNA was obtained with a FastTrack 2.0 mRNA isolation kit (Invitrogen). To select leptin-induced transcripts in the forebrain, cDNA subtraction based on selective suppression of PCR was performed with PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA) following the manufacturer's protocol. Double-stranded cDNAs were synthesized from 2 μg of mRNAs using avian myeloblastosis virus reverse transcriptase and T4 DNA polymerase. cDNAs derived from *ob/ob* mice injected with leptin (tester pool) and cDNAs from *ob/ob* mice injected with PBS (driver pool) were digested with the restriction enzyme RsaI. Two types of adapter, provided by the manufacturer, were independently ligated to the tester cDNAs. Each of the tester cDNA pools was hybridized with excess from the driver cDNA pool and incubated at 68 °C for 9 h (first hybridization). Then, the two samples from the first hybridization was immediately mixed (second hybridization), and the resulting annealed material was amplified by suppression PCR: 27 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 1.5 min. The amplified PCR products were subtractive, which presented the differentially expressed genes in the tester population compared with the driver population, and were ligated into the T/A cloning vector pCRII (Invitrogen). The individual cDNA inserts were sequenced using T7 primer by an automated sequencer (ABI PRISM 310 Genetic Analyzer, PerkinElmer Life Sciences). Sequence homology searches were done using the Basic Local Alignment Tool program against the National Center for Biotechnology Informatics database, which includes entries from GenBank™, the European Molecular Biology Laboratory, and DNA Database of Japan databases. To verify the selective expression in the brain of *ob/ob* mice injected with leptin, we analyzed differential expression of the individual cDNA sequences on Northern blots with total RNA from forebrains of PBS- and leptin-injected *ob/ob* mice.

Cell Culture—The mouse hypothalamic neuronal cell line, GT1-7 (18), a gift from Dr. Pamela L. Mellon (University of California, La Jolla, CA), was grown in DMEM with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (all from Invitrogen). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of Probes for NREBP—In the present study, we prepared two types of fragments of NREBP: a 290-bp EcoRI-SalI cDNA fragment of NREBP (coding region 1–275) and a 654-bp EcoRI-NotI cDNA fragment of NREBP (coding region 4492–5110). These fragments were ligated into pBluescript SK(+) vector (Invitrogen) and linearized by cutting with appropriate restriction enzymes.

The probe for Northern blot analysis was prepared by using Megaprime DNA labeling systems and [^{32}P]dCTP (both from Amersham Biosciences). The radioisotope-labeled probes for *in situ* hybridization histochemistry were prepared by using appropriate RNA polymerases (T7 RNA polymerase for the antisense probe and T3 RNA polymerase for the sense probe) and [^{35}S]dUTP (PerkinElmer Life Sciences). We used two types of probes (coding region 1–275 and 4492–5110) for Northern blot analysis and a radioisotope-labeled probe for *in situ* hybridization histochemistry. Similar results were obtained with both probes. We thus reported results with the probe prepared by using the fragment of the NREBP (coding region 1–275).

Northern Blot Analysis—Northern blot analysis was performed with some modifications as described previously (17). Briefly, at 1 h after PBS or leptin injection, *ob/ob* mice were deeply anesthetized with diethyl ether, and the brains were quickly removed. Total RNA was isolated from mediobasal hypothalami (defined caudally by the mammillary bodies, rostrally by the optic chiasm, laterally by the optic tract, and superiorly by the apex of the hypothalamic third ventricle) by using TRI Reagent. After separation on 1.2% agarose gels containing 2.4% formaldehyde, total RNA was transferred to positively charged nylon membranes (Roche Diagnostics). For the tissue blot analysis, mouse Multiple Tissue Northern blot was obtained from Clontech Laboratories. Then, the membranes were hybridized with the ^{32}P-labeled NREBP probe in a quick hybridization solution (Stratagene, La Jolla, CA) at 68 °C for 2 h.

After washing twice in 2× SSC buffer (1× SSC = 44.6 μmol/l sodium chloride, 5 μmol/l trisodium citrate, pH 7.0) containing 0.1% SDS at 68 °C for 15 min, and once in 0.1× SSC buffer containing 0.1% SDS at 68 °C for 20 min, the membranes were exposed to x-ray films for an appropriate period. The membranes were stripped and rehybridized with probe for 18 S ribosomal RNA or GAPDH.

In Situ Hybridization Histochemistry—*In situ* hybridization histochemistry using radioisotope-labeled probes was carried out as described previously (19). Briefly, at 1 h after PBS or leptin injection, *ob/ob* mice were deeply anesthetized with diethyl ether and transcardially perfused with ice-cold 4% paraformaldehyde in PBS. The brains were quickly dissected and postfixed in the same fixative at 4 °C for 16 h. Then, the brains were immersed in 30% sucrose in PBS, embedded in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA), and frozen rapidly in cold *n*-hexane on dry ice. Frozen sections were cut on a cryostat (6-μm thickness) and stored at −80 °C.

After treatment with proteinase K (Roche Diagnostics), the sections were postfixed in 4% paraformaldehyde, treated with acetic anhydride, and dehydrated with ethanol. The sections were then hybridized with a sense or antisense ^{35}S-labeled NREBP riboprobe at 55 °C for 16 h. After rinsing in 2× SSC buffer containing 10 mM dithiothreitol, the sections were treated with ribonuclease A (10 μg/ml; Wako Pure Chemical Industries, Tokyo, Japan) at 37 °C for 30 min. The high stringency washes were performed in 0.1× SSC buffer at 55 °C for 15 min. After dehydration with ethanol, the sections were submerged in emulsion (NTB-2; Kodak, Rochester, NY), exposed for the appropriate number of days, and developed in D-19 developer (Kodak). The sections were counterstained with Mayer's hematoxylin through the emulsion and examined under dark field lateral illumination microscopy (XF-WFL, Nikon, Tokyo, Japan). The sense cRNA probe failed to hybridize in the brain (data not shown).

To evaluate the expression of NREBP mRNA in the hypothalamus, every fifth section was picked from a series of consecutive hypothalamic sections (6 μm), and three sections per mouse were counted for the arcuate nucleus, VMH, dorsomedial hypothalamic nucleus, lateral hypothalamic nucleus, and paraventricular hypothalamic nucleus. For each section, cells in the hypothalamus were considered positive for NREBP gene expression if five or more silver grains were found overlying the cell bodies.

In Situ Hybridization Histochemistry Combined with Immunohistochemistry—*In situ* hybridization histochemistry combined with immunohistochemistry was performed with some modifications as described previously (20). Briefly, the sections were hybridized with an NREBP riboprobe, followed by incubation with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Then, the sections were incubated with goat anti-GHS-R antibody (diluted at 1:200, catalog no. sc-10362, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 16 h. After washing, they were incubated with biotinylated donkey anti-goat IgG antibody (diluted at 1:400, Jackson ImmunoResearch Laboratories), followed by incubation with HRP-conjugated streptavidin (DAKO, Carpinteria, CA). The peroxidase reaction product was visualized with 0.05% diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2. After the reaction, the sections were submerged in the emulsion and counterstained with Mayer's hematoxylin through the emulsion. The specificity of goat anti-GHS-R antibody for immunohistochemistry was confirmed by using the brain sections of GHS-R$^{-/-}$ mice (supplemental Fig. S1, *A* and *B*).

To evaluate the colocalization of NREBP mRNA and GHS-R in the hypothalamus, every fifth section was picked from a series of consecutive hypothalamic sections (6 μm), and three sections per mouse were counted for the arcuate nucleus and VMH. For each section, cells in the arcuate nucleus and VMH were considered positive for NREBP gene expression if five or more silver grains were found overlying the cell bodies and were considered positive for the protein expression of GHS-R if the cell bodies were stained brown.

Plasmid—A fragment of the promoter region of the GHS-R gene (−734 to −121) in pGL3-Basic vector (21) was provided by Dr. Hidesuke Kaji (Kobe University School of Medicine, Kobe, Japan). Full-length mouse NREBP cDNA was obtained by screening a mouse brain cDNA library (Invitrogen) using a standard technique and was ligated into pCMV-SPORT2 vector (Invitrogen).

In Vitro Mutagenesis—*In vitro* mutagenesis was performed with some modifications as described previously (22). The NRE replacement mutant in the GHS-R promoter was made with a QuikChange site-directed mutagenesis kit (Stratagene). The template DNA (wild-type GHS-R promoter in pGL3-Basic vector) was amplified by using the complementary primer pairs: 5′-GAAGCGGGAGCGTGAG**TTTT**TT**T**TCCGAAGCC-CTGGGC-3′ and 5′-GCCCAGGGCTTCGGA**A**A**A**A**AAA**-CTCACGCTCCCGCTTC-3′ (the sites of the nucleotide changes are in boldface type). The PCR amplification protocol was 95 °C for 2 min and then 12 cycles of 95 °C for 30 s, 64 °C for 1 min, 68 °C for 7 min, and a final 10 min extension at 68 °C. To select the mutation-containing synthesized DNA, the PCR product was treated with DpnI endonuclease, which specifically digests the parental DNA template. The product was then self-ligated by using a DNA ligation kit (version 2.1, Takara Bio, Inc., Tokyo, Japan) at 16 °C for 30 min, followed by the transformation into DH-5α competent cells (Invitrogen). The sequences of the mutated regions in GHS-R promoter were confirmed by using the primer pairs RVprimer3 and GLprimer2 (Promega, Madison, WI), both designed for use with pGL3-Basic vector.

Transient Transfection—Transient transfection was carried out with some modifications as described previously (23). Briefly, GT1-7 cells were plated in 24-well plates at a density of 6×10^4 cells/well for the luciferase assays or plated in six-well plates at a density of 3×10^5 cells/well for Western blot analysis. After incubation in the standard medium for 1 day, the cells were transfected with plasmids of mock or NREBP at indicated concentrations using FuGENE 6 transfection reagent (Roche Diagnostics). For luciferase assays, all transient transfections also included 0.5 μg of the wild type or mutant GHS-R promoter in pGL3-Basic vector and 0.1 μg of *Renilla* luciferase control reporter plasmid (pRL-TK; Promega). All

A

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  1 MAADIEQVFRSFVVSKFREIQQELSSGRSEGQLNGETNPPIEGNQAGDTAASARSLPNEEIVQKIEEVLSGVLDTELRYKPDLKEASRKSRCVSVQTDPT 100
101 DEVPTKKSKKHKKHKNKKKKKKKEKEKKYKRQPEESESKLKSHHDGNLESDSFLKFDSEPSAAALEHPVRAFGLSEASETALVLEPPVVSMEVQESHVLE 200
                  K-rich
201 TLKPATKAAELSVVSTSVISEQSEQPMPGMLEPSMTKILDSFTAAPVPMSTAALKSPEPVVTMSVEYQKSVLKSLETMPPETSKTTLVELPIAKVVEPSE 300
301 TLTIVSETPTEVHPEPSPSTMDFPESSTTDVQRLPEQPVEAPSEIADSSMTRPQESLELPKTTAVELQESTVASALELPGPPATSILELQGPPVTPVPEL 400
401 PGPSATPVPELSGPLSTPVPELPGPPATVVPELPGPSVTPVPQLSQELPGPPAPSMGLEPPQEVPEPPVMAQELSGVPAVSAAIELTGQPAVTVAMELTE 500
                                    son-c repeat
501 QPVTTTEFEQPVAMTTVEHPGHPEVTTATGLLGQPEAAMVLELPGQPVATTALELSGQPSVTGVPELSGLPSATRALELSGQSVATGALELPGQLMATGA 600
601 LEFSGQSGAAGALELLGQPLATGVLELPGQPGAPELPGQPVATVALEISVQSVVTTSELSTMTVSQSLEVPSTTALESYNTVAQELPTTLVGETSVTVGV 700
701 QPLMAQESHMLASNTMETHMLASNTMDSQMLASNTMDSQMLASNTMDSQMLASSTMDSQMLASSTMDSQMLATSTMDSQMLATSSMDSQMLATSSMDSQM 800
                                    son-b repeat
801 LATSSMDSQMLATSSMESQMLASGAMDSQMLASGTMDAQMLASGTMDAQMLASSTQDSAMMGSKSEDPYRLAQDPYRLAQDPYRLGHDPYRLGHDAYRLG 900
901 QDPYRLGHDPYRLTPDPYRVSPRPYRIAPRSYRIAPRPYRLAPRPLMLASRRSMMMSYAAERSMMSSYERSMMSYERSMMSPMAERSMMSAYERSMMSAY 1000
1001 ERSMMSPMAERSMMSAYERSMMSAYERSMMSPMADRSMMSMGADRSMMSSYSAADRSMMSSYSAADRSMMSSYTDRSMMSMAADSYTDSYTDSYTEAYMV 1100
                                    son-a repeat
1101 PPLPPEEPPTMPPLPPEEPPMTPPLPPEEPPEGPALSTEQSALTADNTWSTEVTLSTGESLSQPEPPVSQSEISEPMAVPANYSMSESETSMLASEAVMT 1200
                  P-rich
1201 VPEPAREPESSVTSAPVESAVVAEHEMVPERPMTYMVSETTMSVEPAVLTSEASVISETSETYDSMRPSGHAISEVTMSLLEPAVTISQPAENSLELPSM 1300
1301 TVPAPSTMTTTESPVVAVTEIPPVAVPEPPIMAVPELPTMAVVKTPAVAVPEPLVAAPEPPTMATPELCSLSVSEPPVAVSELPALADPEHAITAVSGVS 1400
1401 SLEPSVPILEPAVSVLQPVMIVSEPSVPVQEPTVAVSEPAVIVSEHTQITSPEMAVESSPVIVDSSVMSSQIMKGMNLLGGDENLGPEVGMQETLLHPGE 1500
1501 EPRDGGHLKSDLYENEYDRNADLTVNSHLIVKDAEHNTVCATTVGPVGEASEEKILPISETKEITELATCAAVSEADIGRSLSSQLALELDTVGTSKGFE 1600
                                    clone 1-42
1601 FVTASALISESKYDVEVSVTTQDTEHDMVISTSPSGGSEADIEGPLPAKDIHLDLPSTNFVCKDVEDSLPIKESAQAVAVALSPKESSEDTEVPLPNKEI 1700
1701 VPESGYSASIDEINEADLVRPLLPKDMERLTSLRAGIEGPLLASEVERDKSAASPVVISIPERASESSSEEKDDYEIFVKVKDTHEKSKKNKNRDKGEKE 1800
1801 KKRDSSLRSRSKRSKSSEHKSRKRTSESRSRARKRSSKSKSHRSQTRSRSRSRRRRRSSRSRSKSRGRRSVSKEKRKRSPKHRSKSRERKRKRSSSRDNR 1900
                                    SR domain
1901 KAARARSRTPSRRSRSHTPSRRRRSRSVGRRRSFSISPSRRSRTPSRRSRTPSRRSRTPSRRSRTPSRRSRTPSRRRRSRSAVRRRSFSISPVRLRRSRT 2000
2001 PLRRRFSRSPIRRKRSRSSERGRSPKRLTDLDKAQLLEIAKANAAAMCAKAGVPLPPNLKPAPPPTIEEKVAKKSGGATIEELTEKCKQIAQSKEDDDVI 2100
2101 VNKPHVSDEEEEEPPFYHHPFKLSEPKPIFFNLNIAAAKPTPPKSQVTLTKEFPVSSGSQHRKKEADSVYGEWVPVEKNGEESKDDDNVFSSSLPSEGRV 2200
2201 KRQGRVKRQMKQPAASHLTVTRCNSLCGTKPQSEKHRIAEKSVITSLPNIGPSMHLWEGSPRYNYLASRFASRLYSSRFWW
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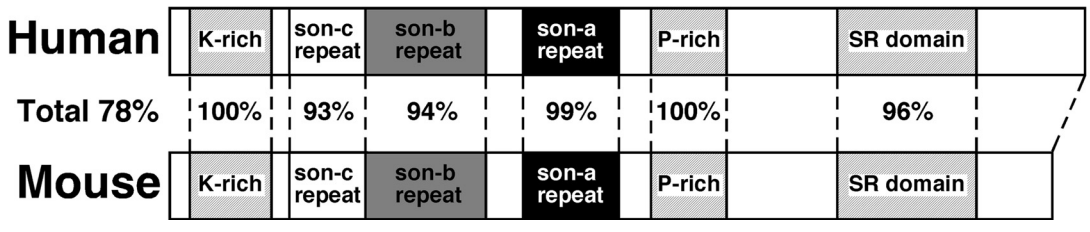
B



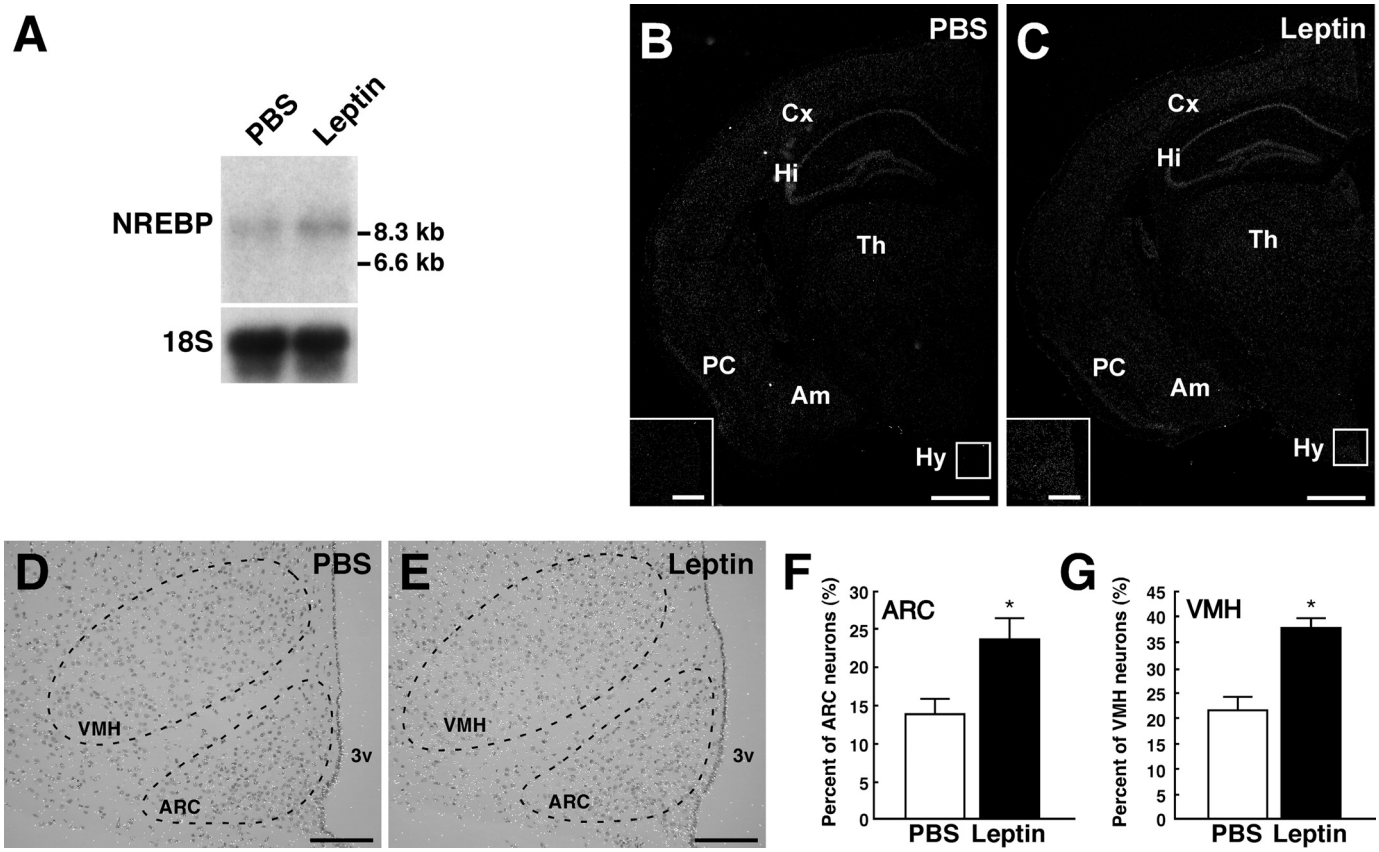


FIGURE 2. **Expression of NREBP in the hypothalamus of PBS- or leptin-injected *ob/ob* mice.** *A*, Northern blot analysis of NREBP mRNA in the hypothalamus of PBS- or leptin-injected *ob/ob* mice. Two micrograms of total RNA isolated from the hypothalamus were separated on agarose gels and then transferred to nylon membranes. The membranes were hybridized with ^{32}P-labeled NREBP probe. The membranes were stripped and rehybridized with probe for 18 S ribosomal RNA to control for loading of the lanes. RNA size markers (in kilobase pairs) are shown to the *right*. *B* and *C*, dark field views of *in situ* hybridization histochemistry for NREBP in the brain of PBS- (*B*) or leptin-injected (*C*) *ob/ob* mice ($n = 4$ per group). The sections were hybridized with ^{35}S-labeled NREBP probe. The *boxed regions* indicated in *B* and *C* are shown at a higher magnification in each inset. *Cx*, cortex; *Hi*, hippocampus; *Th*, thalamus; *PC*, piriform cortex; *Am*, amygdala; *Hy*, hypothalamus. *Scale bars*, 1 mm; 200 μm in *insets*. *D* and *E*, semi-bright field views of *in situ* hybridization histochemistry for NREBP in the hypothalamus of PBS- (*D*) or leptin-injected (*E*) *ob/ob* mice ($n = 4$ per group). The arcuate nucleus and VMH are shown by the *dotted lines*. *ARC*, arcuate nucleus; *3v*, third ventricle. *Scale bars*, 200 μm. *F* and *G*, NREBP-expressing cells in PBS- (*white bar*) or leptin-injected (*black bar*) *ob/ob* mice were quantified as the percentage of positive neurons in the total neurons of the arcuate nucleus (*F*) and VMH (*G*). Data represent the means ± S.E. *, $p < 0.05$ Student's *t* test.

cells were transfected with FuGENE 6 transfection reagent (μl) and DNA (μg) at a ratio of 3:1.

Luciferase Assay—Luciferase assay was performed by using a Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega) with some modifications as described previously (22). Briefly, at 48 h after the transfection, the cells were washed twice with PBS and lysed by passive lysis buffer (Promega). The luciferase activities were defined as the ratio of *Photinus pyralis* luciferase activity from pGL3-Basic derivatives relative to *Renilla reniformis* luciferase activity from pRL-TK, which reflected the efficiency of transfection.

Treatment of Ghrelin in Mock- or NREBP-transfected GT1-7 Cells—At 3 days after the transfection of mock or NREBP, GT1-7 cells were treated with saline or ghrelin (Peptide Institute, Osaka, Japan) dissolved with saline at a dose of 100 nM. Five min after treatment, the cells were used as samples for Western blot analysis.

Western Blot Analysis—Western blot analysis was performed with some modifications as described previously (20). Briefly, at 3 h after PBS or leptin injection, *ob/ob* mice were deeply anesthetized with diethyl ether, and the brains were quickly removed. Lysates from the cultured cells or the mediobasal hypothalami were prepared by using RIPA buffer (Upstate Biotechnology, Lake Placid, NY) containing protease inhibitor mixture (Upstate Biotechnology), 1 mM orthovanadate, 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. The protein concentrations in the lysates were determined by using a BCA protein assay kit (Pierce, Rockford, IL). Ten micrograms of protein from cultured cells or 20 μg of protein from the tissues were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). After blocking with 5% ECL blocking reagent (GE Healthcare) at room temperature for 1 h, the blotted membranes were incubated with rabbit anti-GHS-R antibody (diluted at 1:500, cata-

FIGURE 1. **The structure of mouse NREBP.** *A*, amino acid sequence of mouse NREBP. The *white box*, *shaded box*, and *black box* indicate son-c, son-b, and son-a repeats, respectively. The Lys-rich, Pro-rich, and SR domain are *underlined*. The region that came from cDNA subtraction is *underlined twice* (clone 1-42). *B*, schematic representation of homology between human NREBP and mouse NREBP. The percentage of amino acid homology between corresponding regions of human NREBP and mouse NREBP is indicated.

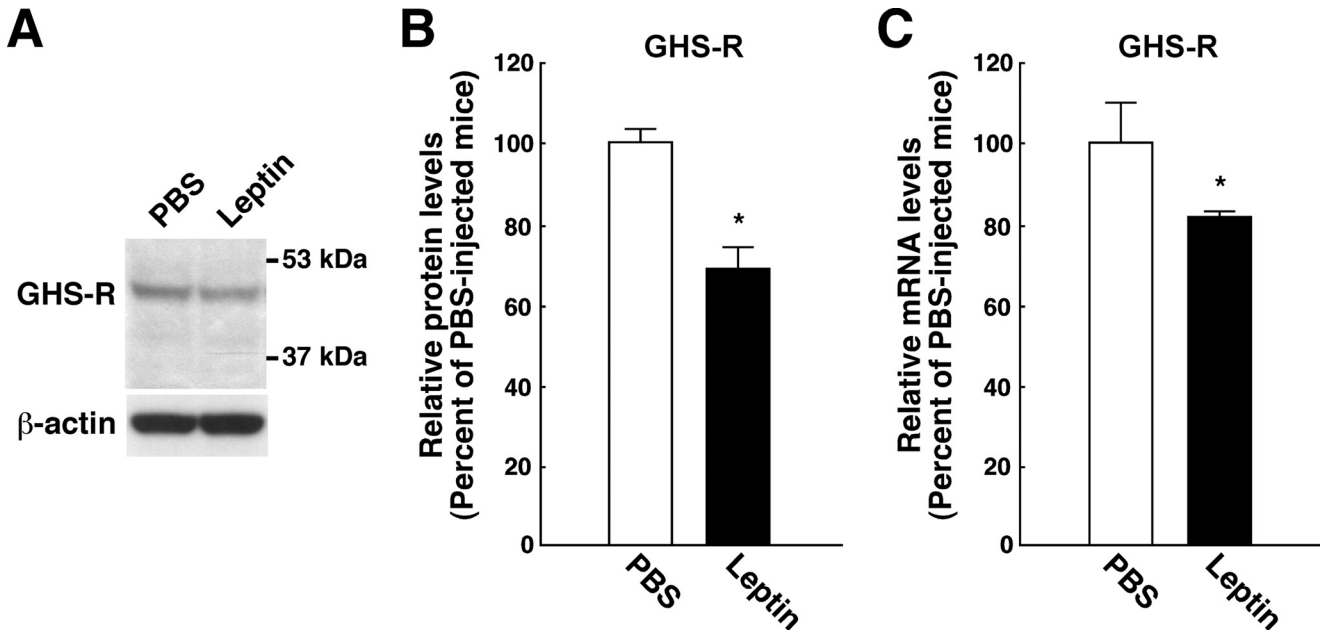


FIGURE 3. **The effect of leptin on the expression of GHS-R in the hypothalamus of *ob/ob* mice.** *A*, Western blot analysis of GHS-R in the hypothalamus of PBS- or leptin-injected *ob/ob* mice ($n = 4$ per group). Lysates prepared from the hypothalamus of PBS- or leptin-injected *ob/ob* mice were separated by SDS-PAGE and immunoblotted with anti-GHS-R antibody. The blots were then stripped and reprobed with anti-β-actin antibody to ensure equal loading of proteins. Apparent molecular masses are indicated on the *right*. *B*, quantitative analysis of the protein expression of GHS-R in the hypothalamus of PBS- (*white bar*) or leptin-injected (*black bar*) *ob/ob* mice. The band intensities of GHS-R were normalized with the band intensities of β-actin and are shown as a percentage relative to the intensities of PBS-injected mice in the bar graphs. *C*, expression of GHS-R mRNA in the hypothalamus of PBS- or leptin-injected mice ($n = 3$ per group). Quantitative real-time PCR was performed by using mRNA prepared from the hypothalamus of PBS- (*white bar*) or leptin-injected (*black bar*) *ob/ob* mice. Data represent the means ± S.E. *, $p < 0.05$ Student's *t* test.

log no. sc-20748, Santa Cruz Biotechnology), rabbit anti-leptin receptor antibody (diluted at 1:500, catalog no. 07–096, Upstate Biotechnology), or rabbit antiphospho-AMP-activated protein kinase α (AMPKα) antibody (diluted at 1:500; Cell Signaling Technology, Beverly, MA) at 4 °C for 16 h, followed by incubation with HRP-conjugated donkey anti-rabbit IgG (diluted at 1:4,000, GE Healthcare). Labeled proteins were detected with chemiluminescence using ECL detection reagent (GE Healthcare) according to the manufacturer's instructions. The membranes were exposed to Hyperfilm ECL (GE Healthcare) for an appropriate period. Then the blotted membranes were stripped in 0.25 M glycine, pH 2.5, at room temperature for 10 min and incubated with mouse anti-β-actin antibody (diluted at 1:10,000; Sigma) or rabbit anti-AMPKα antibody (diluted at 1:500; Cell Signaling Technology) at 4 °C for 16 h, followed by incubation with HRP-conjugated donkey anti-rabbit IgG (diluted at 1:4,000, GE Healthcare) or HRP-conjugated donkey anti-mouse IgG (diluted at 1:20,000, Jackson ImmunoResearch Laboratories) at room temperature for 1 h. The specificity of rabbit anti-GHS-R antibody for Western blot analysis was confirmed by using the hypothalamus of GHS-$R^{-/-}$ mice (supplemental Fig. S1*C*).

Quantitative Real-time PCR—Quantitative real-time PCR was performed with some modifications as described previously (24). Briefly, at 3 h after PBS or leptin injection, *ob/ob* mice were deeply anesthetized with diethyl ether, and the brains were quickly removed. Total RNA was extracted from mediobasal hypothalami as described above. The cDNA from the total RNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The following TaqMan gene expression assays (Applied Biosystems) were used: NREBP (Mm00490912_m1), GHS-R (Mm00616415_m1), and 18 S (Hs99999901_s1). Quantitative real-time PCR for each gene was performed using StepOnePlus real-time PCR system (version 2.0, Applied Biosystems) and TaqMan gene expression master mix (Applied Biosystems). The PCR amplification protocol was 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative abundance of transcripts was normalized by the expression of 18 S mRNA and analyzed using ΔΔCT method.

Statistical Analysis—Results were shown as means ± S.E. Statistically significant differences for the results from Western blot analysis of pAMPKα and AMPKα in GT1-7 cells were determined by analysis of variance followed by a post hoc Bonferroni test. All other results were analyzed by a Student's *t* test. The criterion for statistical significance was $p < 0.05$.

RESULTS

Identification of Mouse NREBP—To identify the novel genes induced by leptin in the hypothalamus, we performed cDNA subtraction based on selective suppression of PCR using mRNAs from the forebrain of leptin-deficient *ob/ob* mice at 1 h after intravenous injection of PBS or mouse leptin. We chose at random 100 clones in the subtractive library, sequencing their inserts to identify and exclude redundant clones. Inducibility of the genes encoded by the inserts was validated by Northern blot analysis, using the insert as a probe (data not shown). Among them, a gene encoded by a clone 1–42 insert was the mouse homolog of human NREBP. Mouse NREBP consisted of 2281 amino acids and showed 78% homology to human NREBP at the amino acid level. The important domains, Lys-rich, Pro-rich, and Ser/Arg domains, were well preserved between

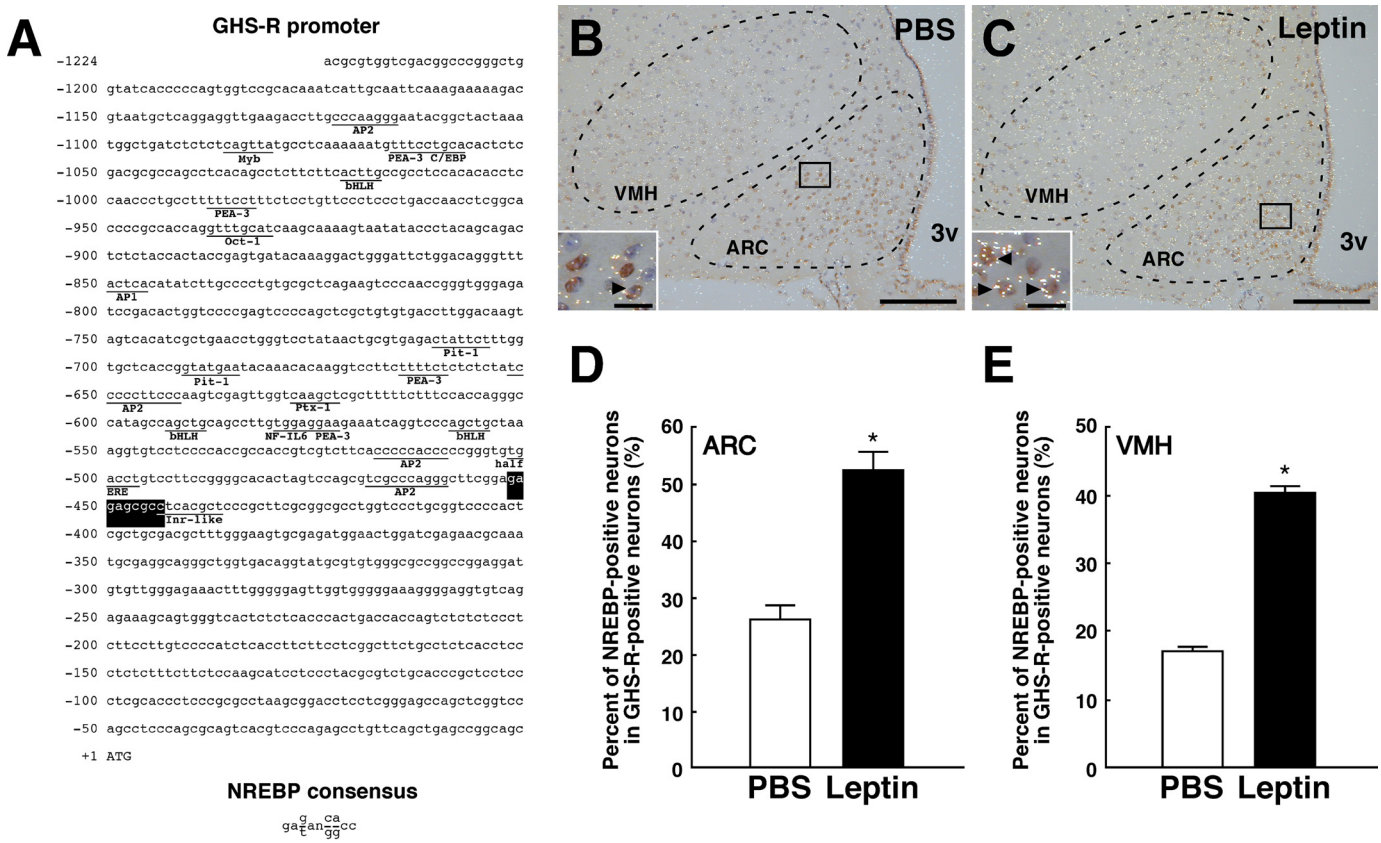


FIGURE 4. **The possibility of a repressive effect of NREBP on the promoter activity of GHS-R.** *A*, optimal NREBP binding sites in the promoter region of GHS-R. The GHS-R promoter region contains a consensus sequence of NRE (from −452 to −444), as shown by the *black box*. *Underlined* sequences indicate the putative binding sites for transcription factors or the initiator-like sequence. *B* and *C*, colocalization of NREBP mRNA and GHS-R in the hypothalamus of PBS- (*B*) or leptin-injected (*C*) *ob/ob* mice ($n = 4$ per group). The *boxed* regions indicated in *B* and *C* are shown at a higher magnification in each *inset*. *Arrowheads* indicate GHS-R-positive neurons (*brown*) with signals for NREBP mRNA (*white dots*). The arcuate nucleus and VMH are shown by the *dotted lines*. *ARC*, arcuate nucleus; *3v*, third ventricle. *Scale bars*, 200 μm; 15 μm in *insets*. *D* and *E*, NREBP- and GHS-R double-positive neurons in PBS- (*white bar*) or leptin-injected (*black bar*) *ob/ob* mice were quantified as the percentage of double-positive neurons in the GHS-R-positive neurons of the arcuate nucleus (*D*) and VMH (*E*). Data represent the means ± S.E. *, $p < 0.05$ Student's *t* test.

human and mouse NREBP (Fig. 1*B*). In addition, the unique repeats, son-a, son-b, and son-c repeats, also showed high homologies in human and mouse: 99% in the son-a, 94% in the son-b, and 93% in the son-c repeat (Fig. 1*B*).

Induction of NREBP by Leptin in Hypothalamus—The intense expression of mouse NREBP mRNA was observed in the brain as well as heart, spleen, liver, skeletal muscle, kidney, and testis (supplemental Fig. S2). To investigate whether leptin up-regulates the expression of NREBP in the hypothalamus, we performed Northern blot analysis using the total RNA from the whole hypothalamus of *ob/ob* mice at 1 h after intravenous injection of PBS or leptin. In the hypothalamus, a major band corresponding to NREBP was detected in PBS-injected *ob/ob* mice (Fig. 2*A*), which increased in the leptin-injected *ob/ob* mice (Fig. 2*A*).

In situ hybridization histochemistry revealed that NREBP mRNA was moderately expressed in some neurons of the brain including the cortex, hippocampus, and piriform cortex in PBS-injected *ob/ob* mice (Fig. 2*B*). However, the expression of NREBP was very weak in the hypothalamus. In addition, the expression of NREBP was increased by leptin exclusively in the hypothalamus at 1 h after leptin administration without affecting NREBP expression in the other regions of the brain (Fig. 2, *B* and *C*).

To determine the localization of leptin-induced NREBP mRNA in the hypothalamus, we measured the number of NREBP-positive cells in the hypothalamus. The number of NREBP-positive cells was increased by leptin in the arcuate nucleus (PBS-injected, 13.7 ± 2.0%; leptin-injected, 23.5 ± 2.7%; Fig. 2, *D*–*F*) and VMH (PBS-injected, 21.4 ± 2.5%; leptin-injected, 37.5 ± 1.9%; Fig. 2, *D*, *E*, and *G*). There were no differences in the expression of NREBP between PBS-injected or leptin-injected *ob/ob* mice in the other hypothalamic regions: dorsomedial hypothalamic nucleus (PBS-injected, 11.8 ± 2.1%; leptin-injected, 12.2 ± 1.3%), lateral hypothalamic nucleus (PBS-injected, 8.4 ± 1.2%; leptin-injected, 9.2 ± 1.2%), and paraventricular hypothalamic nucleus (PBS-injected, 14.9 ± 3.0%; leptin-injected, 13.7 ± 1.7%).

Suppression of GHS-R Expression by Leptin in Hypothalamus—Next, we explored the transcriptional targets of leptin-induced NREBP in the arcuate nucleus and VMH. Particular attention was paid to GHS-R, which is the ghrelin receptor and mainly expressed in the arcuate nucleus and VMH in the hypothalamus. However, it is unclear whether leptin regulates the expression of GHS-R in the hypothalamus. To test this possibility, we examined the expression of GHS-R at the mRNA and protein levels in the hypothalamus of *ob/ob* mice at 3 h after leptin injection. The expression levels of GHS-R mRNA and protein in the hypothalamus of leptin-injected mice were significantly low compared with those in PBS-injected mice (Fig. 3, *A*–*C*),

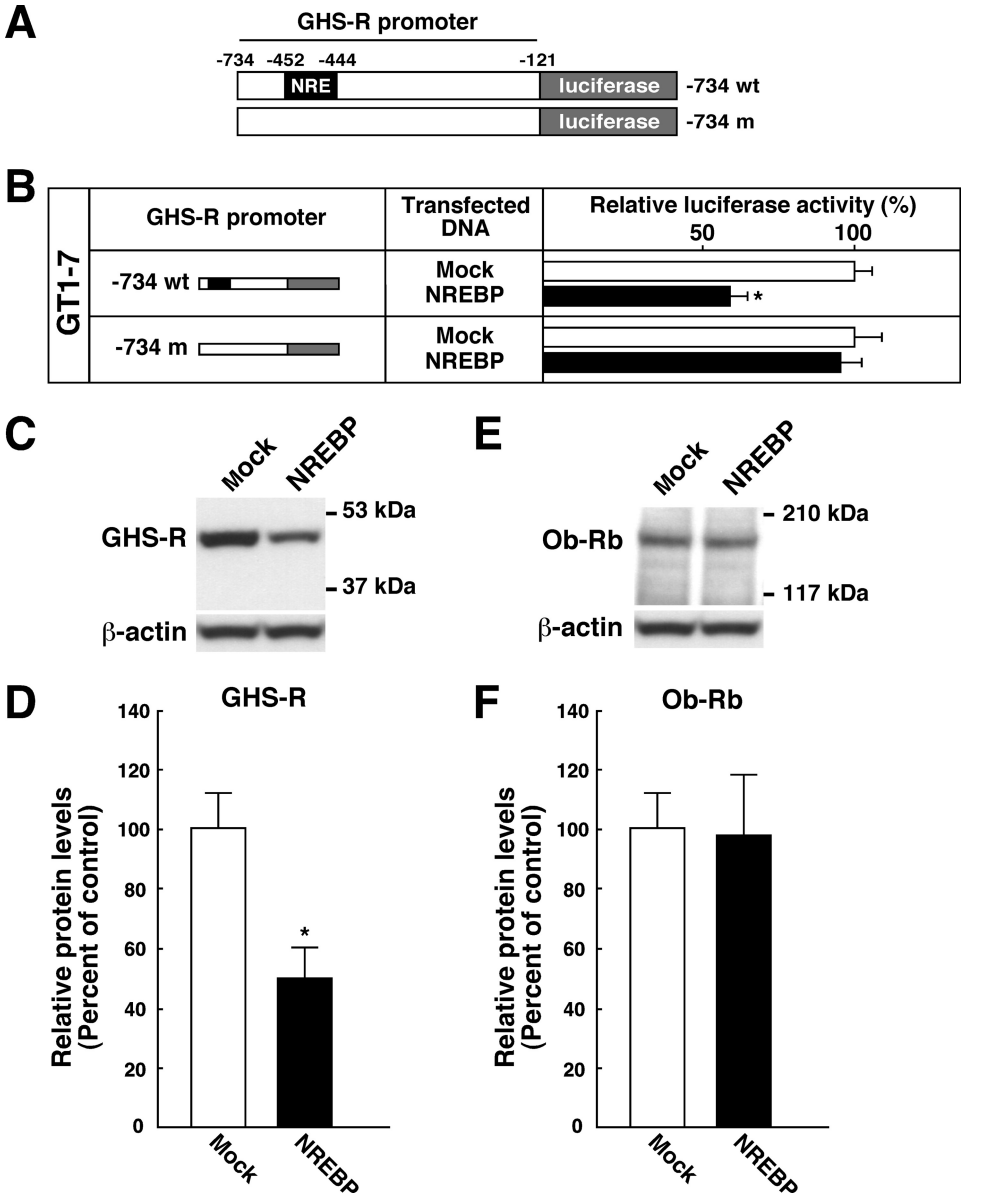


FIGURE 5. **Effects of NREBP on the expression of GHS-R and ob-Rb.** *A*, schematic representation of wild-type GHS-R promoter-luciferase fusion gene (−734 WT) and NRE replacement mutant of GHS-R promoter-luciferase fusion gene (−734 m). *B*, effects of NREBP on the promoter activities of GHS-R in GT1-7 cells. Mock or NREBP (2.5 μg) was transiently transfected with GHS-R promoter-luciferase fusion construct (0.5 μg) and *Renilla* luciferase control reporter plasmid, pRL-TK (0.1 μg) into GT1-7 cells and incubated for 2 days. The promoter activities of GHS-R in mock- (*white bar*) or NREBP-transfected (*black bar*) cells were normalized with *Renilla* luciferase activity and are shown as a percentage relative to the activities of mock-transfected cells. Data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ Student's *t* test. *C–F*, effects of NREBP on the protein expression of endogenous GHS-R (*C* and *D*) or ob-Rb (E and F) in GT1-7 cells. *C* and *E*, Western blot analysis of GHS-R (*C*) or ob-Rb (*E*) in mock- or NREBP-transfected cells. After 3 days of transfection (5.0 μg), cell lysates from mock- (mock) or NREBP-transfected (NREBP) cells were separated by SDS-PAGE and immunoblotted with anti-GHS-R (*C*) or anti-leptin receptor (*E*) antibodies. Then, the blots were stripped and reprobed with anti-β-actin antibody to ensure equal loading of proteins. Apparent molecular masses are indicated on the *right*. *D* and *F*, quantitative analysis of the protein expression of GHS-R after 3 days of transfection with mock or NREBP. The band intensities of GHS-R or ob-Rb in mock- (*white bar*) or NREBP-transfected (*black bar*) cells were normalized with those of β-actin and are shown in the bar graphs as a percentage relative to the intensities of mock-transfected cells. Data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ Student's *t* test.

suggesting that leptin regulates GHS-R expression in the hypothalamus.

Colocalization of NREBP and GHS-R in Arcuate Nucleus and VMH—In the promoter region of human GHS-R (21), there was one NREBP binding sequence, called NRE (25), from −452 to −444 (Fig. 4*A*). In addition, NREBP mRNA was colocalized with GHS-R in the hypothalamus of PBS-injected *ob/ob* mice, and the number of NREBP/GHS-R-double-positive cells was increased by leptin in the arcuate nucleus (PBS-injected, 26.3 ± 5.0%; leptin-injected, 51.9 ± 7.7%; Fig. 4, *B–D*) and VMH (PBS-injected, 17.8 ± 0.6%; leptin-injected, 42.1 ± 0.9% Fig. 4, *B*, *C*, and *E*) at 1 h after the injections. These results strongly suggested that NREBP may play an important role, including transcriptional regulation, in GHS-R-expressing neurons.

Repression of GHS-R Promoter Activity by NREBP in GT1-7 Cells—To examine the effects of NREBP on the promoter activity of GHS-R, we performed luciferase assay using the fusion construct of luciferase reporter and wild-type promoter of GHS-R from −734 to −121 (−734 WT), which contains the NRE from −452 to −444 (Fig. 5*A*). In addition, we used a hypothalamic neuronal cells, GT1-7, to accomplish this aim because GT1-7 cells express GHS-R endogenously. The luciferase activities of GHS-R promoter (−734 WT) were repressed in NREBP-transfected GT1-7 cells compared with those in mock-transfected GT1-7 cells (60.7 ± 6.7%; Fig. 5*B*).

To define the effects of NREBP on NRE, we made an NRE replacement mutant in the GHS-R promoter (−734 m; Fig. 5*A*) and performed luciferase assay. In contrast to −734 WT, the luciferase activity of −734 m was not repressed by NREBP in GT1-7 cells (Fig. 5*B*), suggesting that NREBP represses GHS-R promoter activity by binding to NRE.

Decrease of GHS-R Protein by NREBP in GT1-7 cells—To investigate whether the repression of promoter activity of GHS-R by NREBP leads to the decrease of protein expression of GHS-R, we performed Western blot analysis for endogenous GHS-R in mock- or NREBP-transfected GT1-7 cells. The endogenous protein expression of GHS-R was markedly decreased in NREBP-transfected GT1-7 cells compared with that of mock-transfected GT1-7 cells after 3 days of transfection (49.7 ± 10.4%; Fig. 4, *C* and *D*). There were no significant differences in the protein expression of ob-Rb between NREBP-transfected and mock-transfected cells (Fig. 5, *E* and *F*).

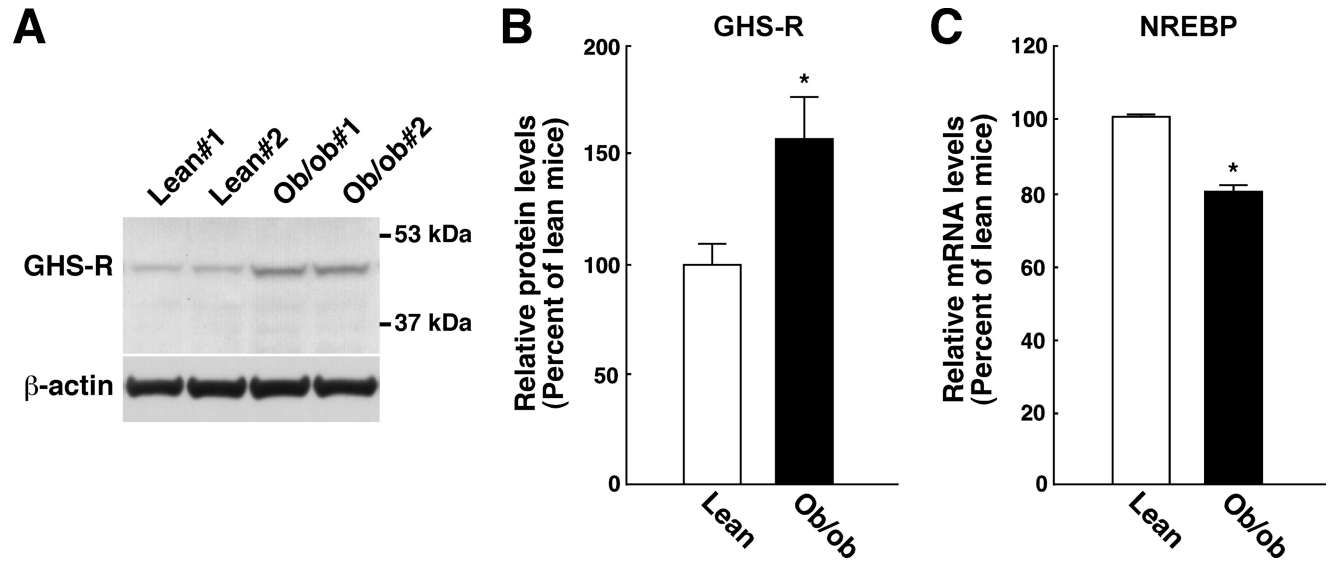


FIGURE 6. **Expression of GHS-R in the hypothalamus of *ob/ob* mice.** *A*, Western blot analysis of GHS-R in the hypothalamus of *ob/ob* mice ($n = 4$ per group). Lysates prepared from the hypothalamus of lean or *ob/ob* mice were separated by SDS-PAGE and immunoblotted with anti-GHS-R antibody. The blots were then stripped and reprobed with anti-β-actin antibody to ensure equal loading of proteins. Apparent molecular masses are indicated on the *right*. *B*, quantitative analysis of the protein expression of GHS-R in the hypothalamus of lean (*white bar*) or *ob/ob* mice (*black bar*). The band intensities of GHS-R were normalized with the band intensities of β-actin and are shown as a percentage relative to the intensities of lean mice in the bar graphs. *C*, expression of NREBP mRNA in the hypothalamus of lean or *ob/ob* mice ($n = 4$ per group). Quantitative real-time PCR was performed by using mRNA prepared from the hypothalamus of lean (*white bar*) or *ob/ob* mice (*black bar*). Data represent the means ± S.E. *, $p < 0.05$ Student's *t* test.

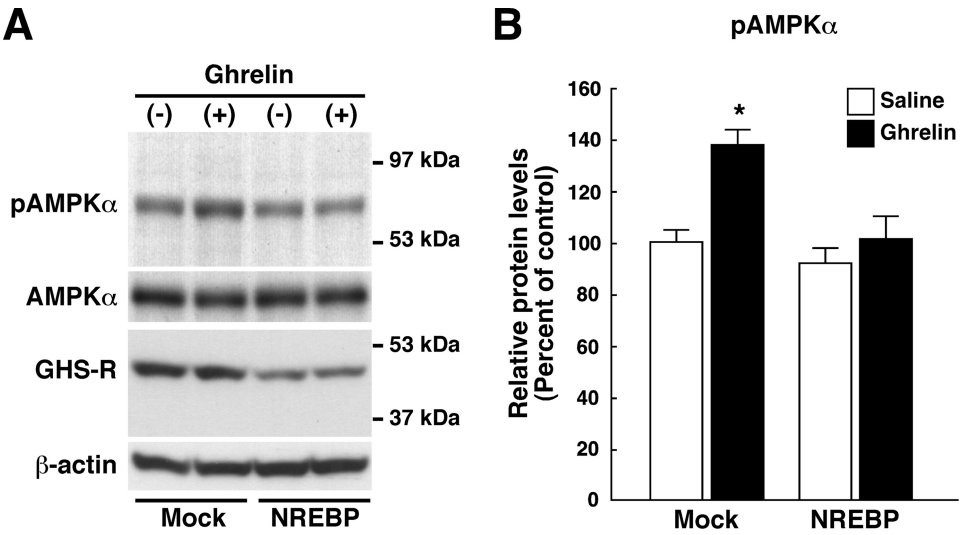


FIGURE 7. **Functional roles of NREBP on the actions of ghrelin.** *A*, inhibitory effects of NREBP on ghrelin-induced AMPK activation in GT1-7 cells. Three days before the experiment, mock or NREBP (5.0 μg) was transiently transfected into GT1-7 cells. The cells were then treated with ghrelin (100 nM) for 5 min. The lysates from these cells were separated by SDS-PAGE and immunoblotted with the anti-pAMPKα or anti-GHS-R antibodies. The blots were stripped and reprobed with anti-AMPKα or anti-β-actin antibody. Apparent molecular masses are indicated on the *right*. *B*, quantitative analysis of the activation of AMPK. The band intensities of pAMPKα of saline- (*white bars*) or ghrelin-treated (*black bars*) cells were normalized with the band intensities of AMPKα and are shown in the bar graphs as a percentage relative to the intensities of mock-transfected saline-treated cells. Data represent the means ± S.E. of three independent experiments. *, $p < 0.05$ analysis of variance followed by post hoc Bonferroni test.

Expression of GHS-R and NREBP in Hypothalamus of ob/ob Mice—To test the effects of leptin-induced NREBP on the expression of GHS-R *in vivo*, we compared the expression of GHS-R between lean and leptin-deficient *ob/ob* mice in the hypothalamus. The expression of GHS-R was increased in the hypothalamus of *ob/ob* mice compared with that of lean mice (Fig. 6, *A* and *B*). In addition, quantitative real-time PCR revealed that the expression of NREBP was decreased in the hypothalamus of *ob/ob* mice compared with that of lean mice (Fig. 6*C*). Thus, the leptin signaling pathway, including NREBP, appear to be important to repress GHS-R expression in the hypothalamus.

Inhibition of Ghrelin-induced AMPK Activation by NREBP in GT1-7 Cells—Recently, hypothalamic AMPK, a key enzyme modulating fatty acid metabolism, is essential for appetite stimulation by ghrelin (26). To determine the effects of NREBP on AMPK activation by ghrelin, we assessed ghrelin-induced phosphorylation of AMPKα in mock- or NREBP-transfected GT1-7 cells. Consistent with Fig. 5 (*C* and *D*), the expression of GHS-R was decreased in NREBP-transfected cells compared with mock-transfected cells (Fig. 7*A*). In mock-transfected GT1-7 cells, ghrelin markedly phosphorylated AMPKα at 5 min after ghrelin treatment (Fig. 7, *A* and *B*). However, phosphorylation of AMPKα was not increased by ghrelin in NREBP-transfected GT1-7 cells (Fig. 7, *A* and *B*).

DISCUSSION

It has been reported that NREBP represses the activities of virus promoters, such as the core promoter of hepatitis B virus (25). Although NREBP expresses in various tissues in human (25) and mouse, the physiological roles of NREBP remain

unclear. In the present study, we identified NREBP as a leptin-induced gene in the mouse hypothalamus. In addition, NREBP repressed the promoter activity of GHS-R via NRE in the hypothalamic neurons. The present study is the first to report the physiological function of NREBP in the hypothalamus.

In the hypothalamic neurons, both transcriptional activities and expression levels of GHS-R were reduced ~45% by NREBP. To confirm whether the ~45% reduction in the expression of GHS-R plays an important role in the signal transduction of ghrelin, we performed functional assays for ghrelin. In hypothalamic neurons, ghrelin activates AMPK by binding to GHS-R (27). In the present study, the overexpression of NREBP completely abolished ghrelin-induced activation of AMPK in a hypothalamic neuronal cell line, GT1-7. Thus, NREBP is important in the regulation of ghrelin signaling, at least in part, through the suppression of GHS-R expression in hypothalamic neurons.

Leptin and ghrelin are inversely correlated in the plasma levels, food intake, and activation of neuropeptide Y neurons (28). Recently, Kohno *et al.* (29) have demonstrated that leptin suppresses ghrelin-induced activation of neuropeptide Y neurons via the phosphatidylinositol 3-kinase- and phosphodiesterase 3-mediated pathway. In addition, the expression of GHS-R is enhanced in the hypothalamus of *fa/fa* rats (30), where normal leptin signaling is ablated by the mutation of leptin receptor, and the GHS-R expression in the hypothalamus is suppressed by leptin treatment (30). However, the molecular mechanisms by which leptin signaling regulates GHS-R expression are largely unknown. In the present study, we also demonstrated that the expression of GHS-R was enhanced in the hypothalamus of *ob/ob* mice. Furthermore, leptin-induced NREBP suppressed the expression and functional roles of GHS-R in the hypothalamus. As the effect of leptin on the expression of GHS-R mRNA takes longer than 2 h (30), suggesting novel gene expression rather than the modulation of signaling pathways, leptin may suppress ghrelin signaling by leptin-induced NREBP.

Both ghrelin gain-of-function and leptin-deficient *ob/ob* mice are hyperphagic and glucose-intolerant (31, 32), suggesting that ghrelin and leptin regulate feeding behavior and glucose metabolism as mutual antagonists. However, it has been reported that the ablation of ghrelin improves the diabetic but not obese phenotype of *ob/ob* mice (33). These findings suggest that factors other than ghrelin are responsible for the development of obesity and hyperphagia in the leptin-deficient *ob/ob* mice. On the other hand, the deficiency of leptin is compensated for by the ghrelin deficiency in the development of diabetes during obesity (33). In the present study, NREBP suppressed ghrelin signaling via the regulation of GHS-R expression. In addition, NREBP was one of the leptin-downstream genes, and its functional abnormality could cause leptin resistance, resulting in insulin resistance. Although further studies are required to determine the precise mechanism of the development of leptin resistance by the mutation of NREBP gene, NREBP can regulate glucose metabolism via linking between leptin and ghrelin signaling and may be an effective target of treatment for diabetes with obesity.

In conclusion, leptin induced the expression of NREBP in the hypothalamus, which suppressed ghrelin signaling via the regulation of GHS-R expression. Our study provides strong evidence for the novel mechanism by which leptin regulates ghrelin signaling in the hypothalamus. Functional abnormality of NREBP may cause leptin resistance, resulting in diabetes with obesity, as obesity is associated with hypothalamic leptin resistance.

Acknowledgments—We thank Dr. Pamela L. Mellon (University of California, La Jolla, CA) for providing the GT1-7 cell line. We also thank Dr. Hidesuke Kaji (Kobe University School of Medicine, Kobe, Japan) for providing the fragment of the 5′-flanking region of the GHS-R gene (−734 to −121) in pGL3-Basic vector. We are grateful to Dr. Dovie Wylie for excellent language support.

REFERENCES

1. Badman, M. K., and Flier, J. S. (2005) *Science* **307,** 1909–1914
2. Coll, A. P., Farooqi, I. S., and O'Rahilly, S. (2007) *Cell* **129,** 251–262
3. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) *Nature* **372,** 425–432
4. Banks, W. A., Kastin, A. J., Huang, W., Jaspan, J. B., and Maness, L. M. (1996) *Peptides* **17,** 305–311
5. Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K. J., Smutko, J. S., Mays, G. G., Wool, E. A., Monroe, C. A., and Tepper, R. I. (1995) *Cell* **83,** 1263–1271
6. Schwartz, M. W., Seeley, R. J., Campfield, L. A., Burn, P., and Baskin, D. G. (1996) *J. Clin. Invest.* **98,** 1101–1106
7. Thornton, J. E., Cheung, C. C., Clifton, D. K., and Steiner, R. A. (1997) *Endocrinology* **138,** 5063–5066
8. Kristensen, P., Judge, M. E., Thim, L., Ribel, U., Christjansen, K. N., Wulff, B. S., Clausen, J. T., Jensen, P. B., Madsen, O. D., Vrang, N., Larsen, P. J., and Hastrup, S. (1998) *Nature* **393,** 72–76
9. Mizuno, T. M., and Mobbs, C. V. (1999) *Endocrinology* **140,** 814–817
10. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K., and Friedman, J. M. (1995) *Science* **269,** 543–546
11. Farooqi, I. S., Jebb, S. A., Langmack, G., Lawrence, E., Cheetham, C. H., Prentice, A. M., Hughes, I. A., McCamish, M. A., and O'Rahilly, S. (1999) *N. Engl. J. Med.* **341,** 879–884
12. Yang, G., Ge, H., Boucher, A., Yu, X., and Li, C. (2004) *Mol. Endocrinol.* **18,** 1354–1362
13. Chen, K., Li, F., Li, J., Cai, H., Strom, S., Bisello, A., Kelley, D. E., Friedman-Einat, M., Skibinski, G. A., McCrory, M. A., Szalai, A. J., and Zhao, A. Z. (2006) *Nat. Med.* **12,** 425–432
14. Münzberg, H., and Myers, M. G., Jr. (2005) *Nat. Neurosci.* **8,** 566–570
15. Howard, J. K., Cave, B. J., Oksanen, L. J., Tzameli, I., Bjørbaek, C., and Flier, J. S. (2004) *Nat. Med.* **10,** 734–738
16. White, D. W., Zhou, J., Stricker-Krongrad, A., Ge, P., Morgenstern, J. P., Dembski, M., and Tartaglia, L. A. (2000) *Diabetes* **49,** 1443–1450
17. Tamura, S., Morikawa, Y., Miyajima, A., and Senba, E. (2003) *Eur. J. Neurosci.* **17,** 2287–2298
18. Mellon, P. L., Windle, J. J., Goldsmith, P. C., Padula, C. A., Roberts, J. L., and Weiner, R. I. (1990) *Neuron* **5,** 1–10
19. Morikawa, Y., Tamura, S., Minehata, K., Donovan, P. J., Miyajima, A., and Senba, E. (2004) *J. Neurosci.* **24,** 1941–1947
20. Komori, T., Gyobu, H., Ueno, H., Kitamura, T., Senba, E., and Morikawa, Y. (2008) *J. Comp. Neurol.* **511,** 92–108
21. Kaji, H., Tai, S., Okimura, Y., Iguchi, G., Takahashi, Y., Abe, H., and Chihara, K. (1998) *J. Biol. Chem.* **273,** 33885–33888
22. Nakano, Y., Furuta, H., Doi, A., Matsuno, S., Nakagawa, T., Shimomura, H., Sakagashira, S., Horikawa, Y., Nishi, M., Sasaki, H., Sanke, T., and Nanjo, K. (2005) *Diabetes* **54,** 3560–3566

23. Hisaoka, T., Morikawa, Y., Komori, T., Sugiyama, T., Kitamura, T., and Senba, E. (2006) *Eur. J. Neurosci.* **23,** 3149–3160
24. Doi, A., Shono, T., Nishi, M., Furuta, H., Sasaki, H., and Nanjo, K. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 885–890
25. Sun, C. T., Lo, W. Y., Wang, I. H., Lo, Y. H., Shiou, S. R., Lai, C. K., and Ting, L. P. (2001) *J. Biol. Chem.* **276,** 24059–24067
26. López, M., Lage, R., Saha, A. K., Pérez-Tilve, D., Vázquez, M. J., Varela, L., Sangiao-Alvarellos, S., Tovar, S., Raghay, K., Rodríguez-Cuenca, S., Deoliveira, R. M., Castañeda, T., Datta, R., Dong, J. Z., Culler, M., Sleeman, M. W., Alvarez, C. V., Gallego, R., Lelliott, C. J., Carling, D., Tschöp, M. H., Diéguez, C., and Vidal-Puig, A. (2008) *Cell Metab.* **7,** 389–399
27. Kohno, D., Sone, H., Minokoshi, Y., and Yada, T. (2008) *Biochem. Biophys. Res. Commun.* **366,** 388–392
28. Nogueiras, R., Tschöp, M. H., and Zigman, J. M. (2008) *Ann. N.Y. Acad. Sci.* **1126,** 14–19
29. Kohno, D., Nakata, M., Maekawa, F., Fujiwara, K., Maejima, Y., Kuramochi, M., Shimazaki, T., Okano, H., Onaka, T., and Yada, T. (2007) *Endocrinology* **148,** 2251–2263
30. Nogueiras, R., Tovar, S., Mitchell, S. E., Rayner, D. V., Archer, Z. A., Dieguez, C., and Williams, L. M. (2004) *Diabetes* **53,** 2552–2558
31. Bailey, C. J., Atkins, T. W., Conner, M. J., Manley, C. G., and Matty, A. J. (1975) *Horm. Res.* **6,** 380–386
32. Bewick, G. A., Kent, A., Campbell, D., Patterson, M., Ghatei, M. A., Bloom, S. R., and Gardiner, J. V. (2009) *Diabetes* **58,** 840–846
33. Sun, Y., Asnicar, M., Saha, P. K., Chan, L., and Smith, R. G. (2006) *Cell Metab.* **3,** 379–386