FoxO1 Inhibits Leptin Regulation of Pro-opiomelanocortin Promoter Activity by Blocking STAT3 Interaction with Specificity Protein 1*

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Leptin controls food intake and energy expenditure by regulating hypothalamic neuron activities. Leptin exerts its actions through complex signaling pathways including STAT3 phosphorylation, nuclear translocation, and binding to target gene promoter/cofactor complexes. Deficient or defective leptin signaling leads to obesity, which may be caused by insufficient leptin levels and/or resistance to leptin signaling. To understand the molecular mechanisms of leptin resistance, we studied the regulation of pro-opiomelanocortin (POMC) gene expression by leptin. We show that phospho-STAT3 activates POMC promoter in response to leptin signaling through a mechanism that requires an SP1-binding site in the POMC promoter. Furthermore, FoxO1 binds to STAT3 and prevents STAT3 from interacting with the SP1-POMC promoter complex, and consequently, inhibits STAT3-mediated leptin action. Our study suggests that leptin action could be inhibited at a step downstream of STAT3 phosphorylation and nuclear translocation, and provides a potential mechanism of leptin resistance in which an increased FoxO1 antagonizes STAT3-mediated leptin signaling.

Leptin, a hormone secreted from adipose tissue, regulates food intake and energy expenditure (1). By a saturated transport mechanism, circulating leptin enters brain through the blood-brain barrier to act on at least two classes of neurons: POMC neurons to promote the production of anorexigenic POMC and neuropeptide Y/Agouti-related peptide neurons to down-regulate the production and secretion of orexigenic neuropeptide Y and Agouti-related peptide (2–4). Leptin exerts its actions through complex signaling pathways upon its binding and activation of the long form leptin receptor (OBRb) but not the other forms of leptin receptors (OBRe, OBRb, OBRc, and OBRd) (5, 6). Activated OBRb turns on Jak2-STAT3 pathway, including STAT3 phosphorylation and translocation into the nucleus, and its eventual regulation of target gene promoter activities, e.g. activation of POMC transcription (7). Plasma and cerebrospinal fluid leptin levels are often higher in obese subjects, as expected from their higher fat volume compared with the lean (8). However, leptin fails to effect downstream physiological consequences in these animals because of impairment in the leptin signaling pathways, collectively referred as leptin resistance (9). The molecular mechanisms underlying leptin resistance are still unclear. One possibility is that increased activity of SOCS3 suppresses STAT3 phosphorylation and activity on its target genes, based on analysis of DIO mice after 14 weeks of high fat diet (HFD) feeding (10). Recent studies using DIO mice after 4–5 weeks on HFD showed that the levels of leptin-stimulated STAT3 phosphorylation were comparable with those of lean mice on a normal chow diet (10, 11). Mice after 4–5 weeks of HFD feeding showed altered metabolism and increased leptin level, indicating that they may be in an early stage of leptin resistance (10). The fact that STAT3 phosphorylation was unchanged at this early stage but was suppressed at late stages of leptin resistance suggests different molecular mechanisms operating during the early and late stages of leptin resistance. For early stages of leptin resistance, because the level of STAT3 phosphorylation was unaltered, the impairment must lie downstream of STAT3 activation, possibly by a transcription factor, such as FoxO1 (12, 13). FoxO1, a member of forkhead box-containing protein O superfamily, is a central signaling molecule involved in many aspects of actions, including growth and proliferation as well as metabolic regulation through protein-DNA or protein–protein interactions (14, 15).

POMC is a key neuropeptide induced by leptin (16). POMC expression is reduced in leptin signaling deficient mouse models, such as ob/ob and db/db mice (17). POMC expression is also reduced in leptin-resistant DIO mice (18). Previous studies have shown that leptin-stimulated POMC gene expression is mediated via STAT3 (19). However, how STAT3 regulates
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POMC gene expression and how this regulation may be inhibited are not clear. In this study, we used an in vitro system and demonstrated that phospho-STAT3 activates POMC promoter activity in response to leptin through a mechanism that requires an SP1-binding site in the promoter of POMC gene. Furthermore, FoxO1 binds to STAT3 and prevents STAT3 from interacting with the POMC promoter-SP1 complex, and consequently, inhibits STAT3-mediated leptin action. Our study suggests that leptin action could be inhibited at a step downstream of STAT3 activation and translocation into the nucleus and provides a potential mechanism of leptin resistance in which an increased FoxO1 antagonizes STAT3-mediated leptin signaling.

EXPERIMENTAL PROCEDURES

Animal Welfare and Diet-treated Animals—All of the experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Agency for Science, Technology and Research Biomedical Sciences Institutes. For analysis of FoxO1 expression during early stages of leptin resistance, C57BL/6 male mice were divided into two groups at wean time, with one group receiving HFD (Research Diet D12492) and the other receiving chow diet (Research Diet D12450B) for 5 weeks.

DNA Constructs—The POMC promoter-luciferase construct (pGL3-POMC) was a generous gift from Dr. Accili (Columbia University), pcDNA3-FLAG-mFoxO1 from Dr. Fukamizu (Japan), pN3-SP1 FL-complete from Dr. Suske (Germany), pXJ40-FLAG-STAT3 was described previously (20). All of the other DNA constructs used in this study, including truncation and mutation constructs based on pGL3-POMC, are described in detail in supplemental Tables S1 and S2.

Cell Culture and Luciferase Assay—Fp-In HEK293 stable cell lines overexpressing OBRa (293-OBRa) or OBRb (293-OBRb) were described previously (21). The cells were cultured in Dulbecco’s minimal essential medium (Invitrogen) containing 10% fetal bovine serum in a 37 °C incubator with 5% CO₂. One day after plating, the cells were transfected with relevant DNA constructs using FuGENE 6 (Roche Applied Science) and rocked at 4 °C for 10 min. The mixture was then centrifuged at 13,000 rpm for 30 s, and high salt buffer (20% glycerol, 420 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 10% glycerol, 0.2% Nonidet P-40, 20 mM NaF, 1 mM dithiothreitol, and 1× complete protease inhibitor (Roche Applied Science) and rocked at 4 °C for 10 min. The mixture was then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was collected as the nuclear extract.

Co-IP—For STAT3-SP1 interaction, 293-OBRb cells were transfected with pXJ40-FLAG-mSTAT3, and followed by 30-min leptin treatment. Nuclear extracts were prepared from the cells and incubated with SP1 antibody for IP. Immunoblotting of the IP samples was performed using phospho-STAT3 antibody (Cell Signaling). 5% of cell lysate used in each co-IP sample was loaded as input.

For STAT3-FoxO1 interaction in 293-OBRb cells, 293-OBRb cells transfected with expression vectors of pXJ40-FLAG-mSTAT3 and pcDNA3-Myc-mFoxO1 were serum-starved and treated with leptin (50 nM) for 30 min then lysed in lysis buffer. ~500 μg of cell lysate was incubated for 2 h with 1 μg of FLAG (Sigma), Myc (Santa Cruz Biotechnology) antibodies, or control IgG, respectively, followed by IP with protein A+G-Sepharose beads (Invitrogen) for 1 h. The immunoprecipitates were washed four times in lysis buffer and subjected to SDS-PAGE and immunoblotting with antibodies against FLAG or Myc. 5% of cell lysate used in each co-IP sample was loaded as input.

For STAT3-FoxO1 interaction using mouse hypothalamic extracts, hypothalami from 14 C57BL/6 mice that were fed with HFD for 5 weeks was homogenized in chilled whole cell buffer containing 20 mM HEPES, pH 7.9, 280 mM KCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and 20 mM NaF, supplemented with 1 mM Na₃VO₄, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. Each IP reaction contains ~8 mg of hypothalamic extracts and 2 μg of anti-STAT3, anti-FoxO1, or control IgG, and proteins were pulled down by protein A+G-Sepharose beads. IP protein complexes and lysates were analyzed by immunoblotting using anti-

Leptin Binding to Stable HEK293 Cells—This was performed in six-well plates as previously described (21). Briefly, 293-OBRa or 293-OBRb cells were grown to ~90% confluence and washed with PBS. The cells were incubated with ~60,000 cpm of murine recombinant 125I-leptin (PerkinElmer Life Sciences) alone or 125I-leptin with an excessive amount of unlabeled leptin (2 μg/well) for 6 h at 4 °C in a final volume of 1 ml of PBS supplemented with 1% (w/v) bovine serum albumin (fraction V, Sigma). At the end of incubation, unbound 125I-leptin was removed by two PBS washes. 1 ml of 1 N NaOH was then added, and radioactivity in the lysate was measured using a Wizard 1470 Automatic Gamma Counter (PerkinElmer Life Sciences).

Nuclear Extract Preparation from 293 Cells—The cells after treatment with leptin or vehicle were washed twice and collected in cold PBS. The cell suspension was centrifuged at 1,300 rpm for 5 min. The resulting pellet was resuspended with hypertonic buffer containing 20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM Na₃VO₄, 10% glycerol, 0.2% Nonidet P-40, 20 mM NaF, 1 mM dithiothreitol, and 1× complete protease inhibitor (Roche Applied Science) and rocked at 4 °C for 10 min. The mixture was then centrifuged at 13,000 rpm for 30 s, and high salt buffer (20% glycerol, 420 mM NaCl, 1 mM Na₃VO₄, 1 mM dithiothreitol, and 1× complete protease inhibitor in hypertonic buffer without Nonidet P-40) was added to resuspend the pellet. After 40 min rocking, the mixture was centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected as the nuclear extract.
FoxO1 (Santa Cruz Biotechnology) or anti-STAT3 (Cell Signaling).

For FoxO1 effects on STAT3-SP1 interaction, pXJ40-FLAG-mSTAT3 and increasing amounts of pcDNA3-Myc-mFoxO1 were transfected into 293-OBRb cells. The cells were harvested for nuclear fractionation after leptin treatment. Binding of STAT3 to SP1 in nuclear extracts was examined by IP with FLAG antibody (for STAT3) and immunoblotting with Myc (for FoxO1) and SP1 antibodies. 

**Immunoblotting**—The cells were lysed in 1× cell lysis buffer (Cell Signaling) containing 1 mM phenylmethylsulfonyl fluoride. Lysate was incubated on ice for 20 min with gentle rocking and centrifuged at 14,000 × g for 10 min at 4 °C. Equivalent amounts of supernatant samples were analyzed by SDS-PAGE and immunoblotting using antibodies against phospho-STAT3; pan-STAT3, FoxO1, SP1, and Myc; FLAG (Sigma); and Myc (polyclonal, Upstate). 

**EMSA**—Two pairs of oligonucleotides: wild type (5′-GAG GCC CGC CGC CCC CCT-3′ and 5′-GAA GGG GGG GGG CGG GC-3′) and SP1-binding site mutant sequence (5′-GAG GCT TGT TGC CCC CCT-3′ and 5′-GAA GGG GAA CAA CGG GC-3′) were annealed and about 100 ng of the probes were labeled with 50 μCi of [32P]dCTP by klenowexo- (New England Biolabs). After labeling, the probes were purified by using G-50 column, and radioactivity was measured with a LS 6500 multi-purpose scintillation counter (Beckman Coulter). 5 μg of nuclear protein was incubated with the probe with 20,000 cpm in DNA-protein loading buffers (50 mM NaCl, 10 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 1 mM MgCl2, 4% Ficoll, 0.5 mM dithiothreitol, and 1× complete protease inhibitor) in a total volume of 12 μl at room temperature for 15 min. The mixture was resolved by 4% PAGE gel in 0.5× TBE, and the gel was dried at 80 °C by using a gel dryer (Bio-Rad) for 2 h. Supersensitive x-ray film (Kodak) was exposed for 48 h at −80 °C and then developed.

**Immunocytochemistry**—293-OBRb cells were transfected with relevant plasmids 1 day after they were plated on poly-l-lysine-coated coverslips. After leptin or mock treatment, the cells were washed with PBS, fixed in PBS containing 4% paraformaldehyde for 10 min, permeabilized in PBS containing 0.15% Triton X-100 for 10 min, and blocked in ICC buffer (3% bovine serum albumin, 3% goat serum, and 0.15% Triton X-100 in PBS) for 1 h at room temperature. The cells were then probed by using STAT3 and FoxO1 antibodies and fluorescence-conjugated secondary antibodies (Invitrogen). The coverslips were mounted on slides and sealed for observation by confocal microscopy.

**FoxO1 Expression Measurements**—FoxO1 mRNA levels were assessed by real time quantitative PCR essentially as previously described (22). Briefly, the hypothalamus was homogenized in 1 ml of TRIzol reagent (Invitrogen), and total RNA was isolated according to the manufacturer’s specifications. Total RNA was further purified using the RNeasy Mini Kit (Qiagen). RNA samples (1 μg) were treated with DNase and reverse transcribed using random hexamer primers (Promega). The cDNAs were then diluted 10 times for PCR analysis in triplicates. Ten micro-liters of reaction mixture contained 5 μl of SYBR green Universal PCR Master Mix, 300 nm specific target gene primers and 2 μl of cDNA. The amplification was performed as follows: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles each at 95 °C for 15 s, and 60 °C for 60 s, with additional dissociation step in the ABI PRISM 7500 sequence detector system. Standard curves were drawn on the basis of the log of the input RNA versus the critical threshold cycle, which allowed for the critical threshold values to be converted to relative RNA concentrations for each sample. Mouse glyceraldehyde-3-phosphate dehydrogenase was used as endogenous control. SYBR green primers were designed by Primer Express software from Applied Biosystems and the sequences were: 5′-GCT GCA ATG GTAT GTA GGA-3′ and 5′-GTC ACA GTC CAA GCG CTC AAT-3′ for FoxO1; 5′-CAA GGT CAT CCA TGA CAA CTT TG-3′ and 5′-GGC CAT CCA CAG TCT TCT GG-3′ for glyceraldehyde-3-phosphate dehydrogenase.

The FoxO1 protein levels were evaluated by Western blotting. Mouse hypothalamic nuclear extracts were prepared essentially the same as that for 293 cells described above. Briefly, mouse hypothalamus was homogenized in the hypotonic buffer and rocked at 4 °C for 10 min. After centrifugation at 2,000 × g for 10 min, the pellet was resuspended in the high salt buffer. After 40 min of rocking, the mixture was centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected as the nuclear extract. Equivalent amount of nuclear extracts were analyzed by SDS-PAGE and immunoblotting using antibody against FoxO1 (Santa Cruz Biotechnology). The same membrane was later reprobed using β-actin antibody for loading normalization. The films were scanned and quantified using a densitometer (Bio-Rad).

**Statistical Analysis**—The data are presented as the means ± S.E. Comparisons of data were made using two-tailed Student’s t test for independent data. The significance limit was set at p < 0.05.

**RESULTS**

**Leptin Regulation of POMC Promoter Activity via STAT3 Activation**—Leptin regulates energy homeostasis mainly through its central action by binding and activating the long form leptin receptor OBRb, but not the other forms (5, 6). We established a HEK 293 cell line with stable expression of OBRb (293-OBRb) as an in vitro system to study leptin regulation of POMC promoter activity. HEK 293 cells overexpressing OBRa (293-OBRa) was used as a negative control. In these cell lines, only a single copy of the gene construct with C-terminal Myc tagging (Fig. 1A, upper panel) was integrated into the genome to ensure consistent expression level of respective receptors. Because the expression level of the receptors in the stable cell lines was not abundant enough for direct detection from cell lysate by Western blotting, we concentrated the proteins by using leptin-coupled beads. OBRa or OBRb could be detected only in respective stable cell lines, but not the control (Fig. 1A, lower panel). To further confirm the expression of OBRa or OBRb and to validate their proper localization and orientation on the cell surface in these cell lines, we incubated cells with 125I-labeled leptin in the presence or absence of excessive unlabeled leptin. 125I-Labeled leptin could bind both 293-OBRa and 293-OBRb to a similar extent (Fig. 1B). Radioactivity of 125I-labeled leptin, indicative of leptin binding, was not detectable in the presence of excessive unlabeled leptin (Fig. 1B). To test
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**FIGURE 1.** STAT3-mediated leptin regulation of POMC promoter activity in a cell-based system. A, diagram (upper panel) depicts leptin receptor constructs stably expressed in the recombinant HEK 293 cells. The solenoid represents plasma membrane. OBRa and OBRb share identical extracellular sequences, including leptin binding sites (shaded area near the plasma membrane). Y denotes the tyrosine residues implicated in leptin signaling and is present only in OBRb. Both constructs are Myc-tagged at their C termini (black area). The lower panel shows expression of leptin receptors in the 293 cells lines. Leptin receptors from lysates of 293-OBRa, 293-OBRb and control (Ctrl) were concentrated by using leptin-coupled CNBr-activated Sepharose beads, and their expression was examined by using Myc antibody. B, 125-I-leptin was mixed with 293-OBRa or 293-OBRb cells with (white columns) or without (gray columns) the addition of excessive amount of unlabeled leptin. The cells were washed, and radioactivity was counted. The results are the means ± S.E., and represent three independent experiments. *, p < 0.01. C, 293-OBRa and 293-OBRb were transfected with pXJ40-FLAG-mSTAT3. The cells were lysed and subjected to 8% SDS-PAGE after 30 min of leptin or mock treatment. Both phospho-STAT3 and pan-STAT3 antibodies were used for protein detection. Note that phospho-STAT3 signal was detected only in leptin-treated 293-OBRb cells, whereas pan-STAT3 signal was evident in all of the samples. D, 293-OBRa or 293-OBRb was transfected with pXJ40-FLAG-mSTAT3, pGL3-POMC, and pCMV-Renilla. 20 h after leptin treatment, the cells were harvested and lysed, and Firefly luciferase activity of the lysate was measured and normalized to Renilla luciferase activity. The results are the means ± S.E. and represent three independent experiments. *, p < 0.01.

Whether 293-OBRb cells could be used as an in vitro system to study leptin regulation of POMC promoter activity, we introduced a plasmid containing the luciferase gene driven by POMC promoter into 293-OBRb and the control 293-OBRa by transient transfection. We used the POMC promoter containing −646 to +65 of the POMC gene, because full promoter activity requires no more than 480 bp of DNA fragment upstream of transcription initiation site (13, 23). Leptin treatment induced STAT3 phosphorylation only in 293-OBRb cells (Fig. 1C). Similarly, leptin-stimulated luciferase activity was only observed in 293-OBRb, but not in 293-OBRa cells (Fig. 1D), consistent with previous findings that only OBRb is capable of leptin signal transduction (6). We also tested leptin-dependent stimulation of the POMC promoter in AtT-20 cells, but the response was significantly less than that observed in 293-OBRb cells (data not shown), consistent with a previous report (19). When transfected with OBRb expressing vectors, 293 cells have been previously proven to be capable of leptin-mediated intracellular signaling in regulating thyrotropin-releasing hormone promoter (24) and, more relevant to the present study, POMC promoter (19). Taken together, 293-OBRb was a suitable system in studying POMC promoter activity regulation by STAT3-mediated leptin signaling.

**FoxO1 Inhibits STAT3-mediated POMC Activity**—In early stages of leptin resistance, the levels of phospho-STAT3 are comparable in mice on HFD with those on normal chow diet, indicating that impairment of leptin signaling lies downstream of STAT3 activation (10). To mimic the early stages of leptin resistance, in which STAT3 phosphorylation was not reduced, we transfected 293-OBRb cells with the amount of STAT3 that resulted in maximal level of leptin-induced POMC promoter activation (data not shown). FoxO1 was previously shown to have antagonistic effects on STAT3-mediated gene regulation (12, 13). We first tested whether FoxO1 expression was altered in early stages of leptin resistance. Both FoxO1 mRNA levels (Fig. 2A) and protein levels (Fig. 2, B and C) in the hypothalamus were increased in mice on HFD for 5 weeks compared with their control group. We then tested whether FoxO1 could interfere with leptin-induced POMC promoter activity by introducing increasing amount of FoxO1 cDNA on the background of constant STAT3 level in 293-OBRb cells (Fig. 3A). FoxO1 expression levels increased proportionally with the increasing amounts of cDNA used for transfection (Fig. 3A). Although leptin-induced STAT3 phosphorylation was not affected by increasing FoxO1 expression, leptin regulation of POMC promoter activity, as indicated by luciferase activity, was abolished at high expression levels of FoxO1 (Fig. 3B). Leptin regulation of POMC promoter activity was not affected when increasing amounts of a similar-sized control protein were introduced (data not shown). These data demonstrated that high levels of FoxO1 could interfere with leptin signaling and suggest that FoxO1 acted at a step downstream of STAT3 activation.

**FoxO1 Inhibits STAT3 Action in the Nucleus**—To further delineate at which step increasing FoxO1 affected leptin signaling, we tested whether FoxO1 suppressed STAT3 translocation into nucleus after leptin activation. 293-OBRb cells were trans-
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To directly visualize the effects of FoxO1 on leptin-induced STAT3 activation and translocation into the nucleus, we performed immunocytochemistry and confocal microscopy on 293-OBRb cells expressing STAT3 alone or STAT3 plus FoxO1. STAT3 signals were mostly cytoplasmic without leptin treatment but concentrated in the nucleus in leptin-treated samples. STAT3 signals were mostly cytoplasmic without leptin treatment but concentrated in the nucleus in leptin-treated samples. Scale bar, 5 μm.

Essential DNA Fragment for Leptin-induced POMC Promoter Activity—To understand how FoxO1 inhibits STAT3-mediated POMC promoter activation, we first studied how STAT3 interacts with POMC promoter. We made a series of deletion mutants in the promoter region of POMC (mutants 1–11; Fig. 5A and supplemental Table S1) on the background of pGL3-POMC (WT; Fig. 5A) to determine the essential sequence for STAT3-mediated leptin activation of POMC promoter activity. Mutant constructs, along with pGL3-POMC, were separately introduced into 293-OBRb cells, and luciferase activity of various POMC promoter constructs with or without leptin treatment was determined. Deletion mutants without DNA fragment between −138 and −88 (mutants 2, 6, and 8) resulted in a loss of leptin regulation of POMC promoter activity, whereas all of the mutants containing this fragment retained leptin reg-
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FIGURE 5. Essential DNA element in the POMC promoter (−646 to +65) mediating leptin regulation of POMC transcriptional activity. A, diagram of wild type (WT) POMC promoter and deletion mutants. Details of all the mutants were described in supplemental Table S1. B, 293-OBb cells were transfected with pXJ40-FLAG-mSTAT3, pGL3-POMC, and pCMV-Renilla. 20 h after leptin treatment, the cells were lysed in passive lysis buffer. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The results are presented as the means ± S.E. and are a representative of at least three independent experiments in triplicate. *, p < 0.05 and with at least 40% increase in luciferase activity by leptin treatment. Ctrl, control.

FIGURE 6. Mutation of SP1-binding site abolishes leptin regulation of POMC promoter activity. A, diagram of pGL3-POMC construct showing wild type (WT) POMC promoter and SP1-binding site mutant sequence in the essential DNA element (−138 to −88). Details of the mutant are described in supplemental Table S1. EMSA probes containing putative SP1-binding site (Probe 1) or point mutations (Probe 2) were synthesized as described under “Experimental Procedures.” The mutations are highlighted in red. B, EMSA with probe 1 or 2 was carried out using nuclear extracts of 293-OBb cells. A nuclear protein bound to Probe 1 (arrow, lanes 1 and 2) but not Probe 2 (lanes 5 and 6). The protein binding was specifically inhibited by an SP1 antibody (lanes 3 and 4). Samples from two independent reactions were loaded to illustrate reproducibility. C, 293-OBb cells were transfected with pXJ40-FLAG-mSTAT3 and pCMV-Renilla, plus pGL3-POMC or mutant 12. 20 h after leptin treatment, the cells were lysed in passive lysis buffer. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The results are presented as the means ± S.E. and are a representative of at least three independent experiments in triplicate. *, p < 0.05.

ulation, including mutant 11 containing only this DNA fragment (−138 to −88) fused directly upstream of POMC promoter TATA box (Fig. 5B), indicating that a DNA element critical to leptin-enhanced POMC promoter activity lies between −138 and −88 bp upstream from the transcription initiation site.

SP1-binding Element Is Necessary for POMC Promoter Activity—Sequence analysis revealed that the DNA element between −138 and −88 contained a consensus binding sequence to SP1 (Fig. 6A), a constitutive transcription factor present in most cell types (25). To verify whether the putative SP1-binding site interacts with SP1, we synthesized probe 1, corresponding to the original sequence, and probe 2, containing mutations in the putative SP1-binding site (Fig. 6A), and performed EMSA with nuclear extracts from 293-OBb cells. A nuclear protein bound specifically to probe 1, but not to probe 2, and the binding to probe 1 was specifically inhibited by an SP1 antibody (Fig. 6B), but not by STAT3 or FoxO1 antibodies (data not shown). These data indicated that the bound nuclear protein was SP1 and that SP1 and probe 1 formed a specific complex. To examine the potential function of the SP1-binding site in POMC promoter activity, we generated mutant 12, which contained point mutations within the SP1-binding site (Fig. 6A). Functional analysis of mutant 12 in 293-OBb cells revealed that the promoter activity as well as its regulation by leptin were abolished (Fig. 6C), suggesting that leptin-mediated transcriptional activation of POMC promoter was dependent on SP1.

Leptin-mediated POMC Promoter Activity Requires a Direct Interaction of STAT3 and SP1—The lack of STAT3 binding consensus sequence in the DNA element between −138 and −88 suggested that STAT3 regulation of POMC promoter activity was through a way other than direct STAT3-DNA interaction, i.e. STAT3 acted through an intermediate protein to mediate leptin action. Because leptin-induced POMC promoter activity was dependent on SP1, we hypothesized that STAT3 regulated POMC promoter through its interaction with SP1. Co-IP using SP1 antibody resulted in abundant phospho-STAT3 signal in samples from leptin-treated, but not control 293-OBb cells, whereas the control antibody did not pull down phospho-STAT3 from either leptin-treated or control cells (Fig. 7A). These data indicated that SP1 could bind to phospho-STAT3 specifically and further suggested that STAT3 could act through SP1 to mediate leptin regulation of POMC promoter activity.

FoxO1 Inhibits STAT3-SP1 Interaction—Inhibition of STAT3-mediated leptin regulation of POMC promoter activity by FoxO1 occurred at a step downstream of STAT3 translocation into the nucleus (Fig. 4). Given that leptin action required a direct interaction of STAT3 and SP1, we examined whether FoxO1 could interfere with STAT3-SP1 complex formation and thus prevent STAT3 from acting on the POMC promoter.
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We first tested whether FoxO1 could bind to STAT3 by using co-IP of samples from 293-OB-Rb cells. FoxO1 was specifically co-immunoprecipitated in samples treated with antibody (anti-FLAG) against STAT3 and anti-FLAG but not the control antibody (Fig. 7B). Conversely, STAT3 was pulled down in samples treated with antibody (anti-Myc) against Myc-tagged FoxO1, but not the control antibody (Fig. 7C). To confirm FoxO1 and STAT3 interaction under a more physiological condition, we performed two-way co-IP using mouse hypothalamic extracts isolated from mice on HFD for 5 weeks. FoxO1 was specifically pulled down in samples treated with STAT3 antibody (Fig. 7D), whereas STAT3 was precipitated in samples treated with FoxO1 antibody (Fig. 7E). Thus, the two-way co-IP experiments in both 293-OB-Rb and mouse hypothalamic samples confirmed FoxO1-STAT3 binding. Next, we tested whether increasing amount of FoxO1 could reduce and even abolish SP1 binding to STAT3 by co-IP of 293-OB-Rb cells that were transfected with increasing amounts of FoxO1 cDNA. The ability of STAT3 antibody to pull down SP1 was inhibited by FoxO1, and STAT3-SP1 binding was undetectable at high FoxO1 expression levels (Fig. 7F). Together, these data demonstrated that FoxO1 could prevent STAT3-SP1 complex formation by binding to STAT3.

DISCUSSION

Leptin is an adipocyte-derived cytokine that regulates energy homeostasis (1, 5). Leptin deficiency or defective leptin signaling results in massive obesity (9). Although administration of recombinant leptin can reverse the phenotypes associated with lack of leptin production in humans and mice, the pharmaceutical significance of leptin is limited in the majority of obese subjects (5, 26, 27). In most of the obese humans and animals, circulating leptin is often higher than their lean counterparts, but the body fails to respond to leptin because of impaired leptin signaling or leptin resistance (8, 10). DIO mice are often used as an animal model to study leptin resistance (28, 29). In DIO mice, after 14 weeks of high fat diet feeding, the mice show significantly increased body weight and fat mass and high circulating leptin (30). Leptin resistance at this stage is attributed to down-regulated STAT3 activation level by SOCS3 (30, 31). In contrast, DIO mice after 5 weeks of HFD feeding show normal leptin-induced STAT3 phosphorylation, although they also exhibit signs of leptin resistance (10, 11). The unaltered leptin-induced STAT3 phosphorylation in the early stages of leptin resistance indicates that the impairment of leptin signaling occurs at a step downstream of STAT3 phosphorylation (10).

To understand how STAT3 signaling may be inhibited downstream of its activation, we first established a cell-based system to investigate how STAT3 mediates leptin regulation of gene expression. The cell-based system includes stable expression of OBRb and transient expression of Firefly luciferase under the POMC promoter. We chose POMC promoter to study STAT3-mediated leptin regulation for the following reasons: 1) POMC is a key anorexigenic neuropeptide that is regulated by leptin and STAT3 (19) and 2) POMC expression is reduced in leptin-resistant DIO mice (18). We first validated the cell-based system that leptin could stimulate POMC promoter activity through STAT3 activation and then set up a condition to mimic the early stages of leptin resistance, in which POMC promoter activity was suppressed at normal STAT3 level by increasing FoxO1 level. We focused on the structure of POMC promoter to identify the DNA fragment of POMC promoter responsible for normal leptin responsiveness. 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Inhibition of POMC Expression by FoxO1

We also determined how FoxO1 inhibits POMC promoter activity. FoxO1 did not interfere with STAT3 phosphorylation, or STAT3 translocation into the nucleus based on immunoblotting results that nuclear STAT3 level was similar regardless FoxO1 levels, and immunocytochemistry results that nuclear STAT3 signal was indistinguishable in the presence or absence of FoxO1 (Fig. 4). Furthermore, FoxO1 could inhibit STAT3-mediated promoter activity of mutant 4, which lacks the FoxO1-binding site, indicating that FoxO1 inhibition of STAT3 action was not dependent on FoxO1 binding to POMC promoter.4 These findings are consistent with the notion that FoxO1 inhibits STAT3 action by preventing STAT3 from interacting with the SP1-DNA complex by sequestering STAT3. This prompted us to study whether STAT3 interacts with FoxO1 and whether FoxO1 could hinder or even abolish STAT3 interaction with SP1-DNA complex. We performed two-way co-IP, demonstrating that FoxO1 could associate with STAT3 (Fig. 7, B–E), and competition co-IP, revealing that STAT3-SP1 binding was decreased to undetectable level with increasing amount of FoxO1 (Fig. 7F). Together, these data support our proposed model of a potential mechanism how FoxO1 inhibits leptin regulation of POMC promoter; with increasing amount of FoxO1 expression, FoxO1 binds to phosphorylated STAT3 in the nucleus, prevents STAT3 from interacting with the SP1-POMC promoter complex, and, consequently, inhibits STAT3-mediated leptin activation of POMC promoter (Fig. 8B).

Genetic studies in animal models and studies in cell culture systems provide compelling evidence that FoxO proteins play an important role in mediating the effects of insulin and growth factors on gene expression and metabolism and that alterations in FoxO expression could contribute to the development of metabolic aberrations in diabetes (14). The present study provides a new role for a FoxO protein in regulating leptin signaling and in obesity development. Future studies using transgenic mice overexpressing FoxO1 in POMC neurons will shed light and validate our model on the role of FoxO1 in leptin resistance. FoxO1-mediated leptin resistance may be a direct result of its expression up-regulation by pathophysiological factors but may also be due to increased nuclear accumulation of FoxO1, which may result from impaired insulin signaling. It is tempting to speculate that FoxO1 may serve as a link to the so-called “diabetes.”

In summary, we demonstrated that 1) phospho-STAT3 activates POMC promoter in response to leptin signaling through a mechanism that requires the SP1-binding site in the POMC promoter; 2) leptin action can be inhibited by FoxO1 at a step downstream of STAT3 phosphorylation and translocation into the nucleus; and 3) FoxO1 binds to STAT3 and prevents STAT3 from interacting with the SP1-POMC promoter complex and, consequently, inhibits STAT3-mediated leptin action. Our study provides a potential mechanism for leptin

4 G. Yang and W. Han, unpublished observation.
resistance, in which an increased FoxO1 antagonizes STAT3-mediated leptin signaling by blocking the STAT3-SP1 target gene promoter complex formation.

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