Published in final edited form as:

J Neurochem. 2009 July ; 110(1): 390–399. doi:10.1111/j.1471-4159.2009.06144.x.

Melanocortin potentiates leptin-induced STAT3 signaling via MAPK pathway

Yan Zhang¹, Xiaojun Wu¹, Yi He¹, Abba J. Kastin, Hung Hsueh, Charles I. Rosenblum, and Weihong Pan

Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA

Abstract

The co-existence of receptors for leptin and melanocortin in cerebral microvessels suggests possible interactions between leptin and α -melanocyte stimulating hormone (MSH) signaling. In this study, we showed that ObRb and melanocortin receptor MC3R and MC4R were present in enriched cerebral microvessels. To test the interactions between ObRb and MC3R or MC4R-mediated cellular signaling, we over-expressed these plasmids in RBE4 cerebral microvascular endothelial cells and HEK293 cells in culture. Activation of signal transducers and activators of transcription-3 (STAT3) in response to leptin was determined by western blotting and luciferase reporter assays. Production of cAMP downstream to melanocortin receptors was determined with a chemiluminescent ELISA kit. α MSH, which increased intracellular cAMP, also potentiated leptin-induced STAT3 activation. This potentiation was abolished by a specific MEK inhibitor, indicating the involvement of the mitogen-activated kinase pathway. Reversely, the effect of leptin on α MSH-induced cAMP production was minimal. Thus, melanocortin specifically potentiated STAT3 signaling downstream to ObRb by crosstalk with mitogen-activated kinase. The cooperation of ObRb and G protein-coupled receptors in cellular signaling may have considerable biological implications not restricted to feeding and obesity.

Keywords

cAMP; Leptin; MAPK; melanocortin receptors; ObRb; STAT3

Both leptin and α -melanocyte stimulating hormone (α MSH) are anorexigenic peptides. Leptin is a non-glycosylated polypeptide produced mainly in peripheral adipose tissue (Zhang *et al.* 1994; Campfield *et al.* 1995; Murakami and Shima 1995), whereas α MSH is a hormone generated in the hypothalamus and neurointermediate lobe of the pituitary from proopiomelanocortin through post-translational processing (Pritchard *et al.* 2002; Coll *et al.* 2004). Cerebral microvessels express high levels of the leptin receptor (ObR)-a and a low level of ObRb (Hileman *et al.* 2002; Pan *et al.* 2008b). They also show abundant MC3R and MC4R, as we confirm in the Results section of this study. These preliminary findings led to the question of how leptin and α MSH could interact with each other in cellular signaling at the level of the blood—brain barrier (BBB).

ObR is a single transmembrane protein that belongs to the class I cytokine receptor superfamily (Baumann *et al.* 1996; Ghilardi *et al.* 1996; Fruhbeck 2006). Leptin binding induces activation

of Janus Kinase2 (JAK2) and signal transducers and activators of transcription (STATs), particularly STAT3 (Bjørnbæk *et al.* 1997; Bjørnbæk and Kahn 2004; Myers 2004). Among the splicing variants of leptin receptors, only the long form - ObRb - induces STAT3 activation. By contrast, the receptor for α MSH has seven transmembrane domains and is coupled to stimulatory G proteins. Five subtypes of the melanocortin receptors have been characterized, among which MC3R and MC4R are widely expressed in different regions of the brain (Shimizu *et al.* 2007). As G protein-coupled receptors (GPCR), one of the major signaling pathways mediated by MC3R and MC4R is activation of adenylate cyclase with increased intracellular cAMP (Mountjoy *et al.* 1992; Lee *et al.* 2001).

After its production by adipocytes, circulating leptin can cross the BBB (Banks *et al.* 1996; Pan and Kastin 2007a) and blood—cerebrospinal fluid (CSF) barrier (Zlokovic *et al.* 2000). Leptin can also act at the median eminence and area postrema, circumventricular organs outside the BBB, and subsequently affect CNS functions (Kastin and Pan 2006). Interestingly, leptin also facilitates the BBB permeation of the anorexigenic peptide urocortin, which binds to corticotropin-releasing hormone (CRH) receptors (CRHR1 and CRHR2). At the BBB, leptin activates the saturable transport system for urocortin (Kastin *et al.* 2000, 2002; Pan *et al.* 2004). *In vitro* studies also show that the endocytosis of urocortin is facilitated by leptin (Tu *et al.* 2007a), and that both CRHR1 and CRHR2 participate in the transport (Tu *et al.* 2007c). Moreover, urocortin in turn potentiates leptin-induced STAT3 activation (Pan *et al.* 2007). This suggests that an anorexigenic peptide signaling through a GPCR may potentiate the potent STAT3 activation induced by ObR. In this study, we sought to determine the possible crosstalk of leptin and α MSH at the level of cellular signaling.

Materials and methods

Cell culture and reagents

RBE4 cerebral microvessel endothelial cells were provided by Dr Pierre-Olivier Couraud (Institute of Cochin, Paris, France) and cultured as described previously (Roux *et al.* 1994; Roux and Couraud 2005; Yu *et al.* 2006). Human HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Mouse α MSH (Phoenix Pharmaceuticals, Belmont, CA, USA) and leptin (R&D Systems, Minneapolis, MN, USA) were applied to cultured cells over-expressing leptin and melanocortin receptors. Forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), and other agents were purchased from Sigma (St. Louis, MO, USA). PD98059 (Calbiochem, San Diego, CA, USA), the specific inhibitor for mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/MEK) was dissolved in dimethylsulfoxide (DMSO) to prepare a 50 mM stock solution.

End-product RT-PCR

Total RNA was extracted from freshly isolated cerebral microvessels from C57/B6 mice, and reversely transcribed as described previously (Pan *et al.* 2006, 2008b; Yu *et al.* 2007). The expression of MC3R and MC4R in microvessel tissue and cell culture samples was examined by RT-PCR with 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min, followed by 7 min extension at 72°C. The sequences of the PCR primers are shown in Table 1.

Plasmids

Full-length mouse ObRb cDNA in a pcDNA3.1(−) expression vector was obtained from Dr Christian Bjorbaek (Harvard Medical School). STAT3 pAH-Luc luciferase reporter plasmid originated from the Rosenblum Lab as described previously (Rosenblum *et al.* 1996). With mouse hypothalamic cDNA as the template, full-length MC3R cDNA was PCR-amplified with high fidelity AccuPrime™ Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Full-length

mouse MC4R cDNA was purchased from Open Biosystem (Huntsville, AL, USA), and amplified by PCR via pfu native DNA polymerase with proofreading activity. The cloning primers for mouse MC3R and MC4R are shown in Table 2. The restriction enzyme sites *NheI* and *BamHI* were included for both genes. The Kozak sequence (underlined) was included immediately upstream of start codon ATG to ensure efficient translation of the recombinant proteins. Full-length MC3R and MC4R, verified by sequencing, were subcloned into the *NheI* and *BamHI* sites of the mammalian expression vector pcDNA3.1 (-).

Transfection

Triplicate wells of HEK293 and RBE4 cells were transfected at 90% confluency by using Lipofectamine 2000 transfection reagent (Invitrogen). A pcDNA group was included as a negative control for single transfection, and pcDNA was also included to ensure that an equal number of DNA molecules was introduced into the cells when single receptor expression was studied along with the dual receptor expression assays. A dose of 0.8 µg/well of plasmids was introduced to the 24-well plates for RNA analyses, and 4 µg/well was used for the 6-well plates for protein assays. All the luciferase reporter assays also included a Renilla luciferase vector and pAH-luc-Stat3 reporter gene plasmid.

Real-time RT-PCR (qPCR)

qPCR for ObRb mRNA was performed to determine whether co-transfection of MC3R or MC4R affected the level of expression of ObRb. Four groups each of HEK293 and RBE4 cells were studied separately ($n = 3/\text{group}$). The cells were seeded on 24-well plates and transfected with the following: pcDNA3.1 only (group 1), pcDNA3.1 + ObRb (group 2), MC3R + ObRb (group 3), or MC4R + ObRb (group 4). Twenty-four hours later, the cells were collected in RNA lysis buffer containing 1% β-mercaptoethanol. Total RNA was extracted with an RNeasy mini kit (Qiagen, Valencia, CA, USA). After digestion with Dnase I to eliminate trace amounts of DNA contamination, the total RNA was purified with an RNA cleanup kit (Zymo Research, Orange, CA, USA) and quantified at 260 nm with a Bio-Rad spectrometer (Hercules, CA, USA). Reverse transcription of the total RNA was conducted with a High Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed by mixing cDNA samples with Taqman® universal PCR master Mix (Applied Biosystems) and amplified on an ABI 7900 instrument. Standard curves for quantification were generated with template plasmids containing fragments of the respective target genes. The level of expression of the target genes was normalized to that of glyceral-dehydres-3-phosphate dehydrogenase (GAPDH) in the same sample. The primers and probes for mouse ObRb that was transfected and the housekeeping gene GAPDH for the respective host cells are listed in Table 3. Significant differences were determined by one-way ANOVA followed by Tukey's *post-hoc* test.

Western blotting

Western blotting was performed to determine whether co-transfection of MC3R or MC4R with ObRb affected pSTAT3 signaling. Four groups of HEK293 cells were studied 24 h after transfection as described above ($n = 3/\text{group}$). The cells were starved for 12 h, and stimulated with 30 nM of leptin for 1 h. Later, the cells were washed with cold phosphate-buffered saline, and lysed in ice-cold Cell Lytic buffer (Sigma) containing complete protease inhibitor cocktail (Pierce, Rockford, IL, USA). The lysates were sonicated and cleared by ultracentrifugation. The protein content was measured by bicinchoninic acid assay (Pierce). Twenty-five micrograms of protein was electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20, and probed with rabbit anti-pSer⁷²⁷-STAT3 (polyclonal, 1 : 500, Biosource, 44-384G) and rabbit anti-

pTyr⁷⁰⁵-STAT3 (polyclonal, 1 : 100, Santa Cruz Biotechnology (Santa Cruz, CA, USA) sc-7993-R and mouse anti- β -actin (monoclonal, 1 : 10 000, Sigma, A2228) overnight at 4 °C. After thorough wash, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The signals were developed with enhanced chemiluminescence (ECL) plus WB detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

STAT3 luciferase assay

HEK293 and RBE4 cells were serum-starved at 6 h post-transfection, and were treated with leptin, α MSH, or both for 6 h at 18 h post-transfection. For studies with the MEK inhibitor, serum-starved HEK293 cells were incubated with 50 μ M of PD98059 or 0.1% DMSO for 1 h before undergoing the above-mentioned ligand treatment in the presence of the inhibitor. Luciferase assays were performed as described previously (Pan *et al.* 2007). The luminescent intensity of firefly luciferase was normalized to that of Renilla luciferase.

cAMP assay

HEK293 cells were grown in 24-well plates and transfected as described above ($n = 3/\text{group}$). Twenty four hours after transfection, the cells were pre-treated with 300 μ M IBMX for 30 min, followed by α MSH, leptin, or both for 30 min in the presence of IBMX according to the group design detailed in the Results section. cAMP concentrations were measured with a chemiluminescent ELISA kit (Applied Biosystems), as described previously (Tu *et al.* 2007b). The concentration of cAMP was normalized to cell number determined with a Cell Counting Kit 8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, USA).

Statistical analyses

Group means are expressed with their standard errors. ANOVA and Tukey's post-hoc tests were performed with the Statistical Program for Social Sciences (SPSS Inc., San Diego, CA, USA). Graphics were generated with Prism GraphPad software (San Diego, CA, USA).

Results

Identification of suitable cellular systems to study the interactions of ObRb with melanocortin receptors

In enriched cerebral microvessels obtained from normal C57/B6 mice, RT-PCR showed that both MC3R (972 bp) and MC4R (999 bp) mRNAs were present (Fig. 1, left panel). The PCR product was absent in samples in which reverse transcriptase was omitted, and showed the same size in the positive controls with plasmid cDNA. Concurrent with the known presence of ObRb mRNA in cerebral microvessels, as shown in our previous publications (Pan *et al.* 2008a,b), the presence of MC3R and MC4R provided a basis for potential interactions of leptin and α MSH at the BBB level.

We then identified cell lines with a low abundance of endogenous receptors that would be suitable for over-expression studies. In rat cerebral microvascular RBE4 cells of BBB origin, only low copies of MC3R and MC4R were present as shown by rat-specific primers. The specificity of the PCR product was evident by the absence of signal in the negative controls without reverse transcriptase. The PCR products were of the same size as the positive controls in which MC3R or MC4R plasmids were used as the templates (Fig. 1, middle panel). In HEK293 cells originating from human embryonic kidney, MC4R was abundant but MC3R was sparsely present, as measured with human-specific primers (Fig. 1, right panel).

Identification of appropriate doses of α MSH to induce cAMP

To identify the optimal dose range of α MSH in our cellular system, we first tested nine groups of HEK293 cells ($n=3$ /group). At 24 h after seeding to 24-well plates, the cells were transfected with pcDNA3.1, MC3R, or MC4R plasmids (0.2 nM/well, about 0.5 μ g in 0.6 mL). Twenty hours later, the cells were treated with 0.01, 0.1, 1, or 10 nM of α MSH for 30 min. IBMX was included during the entire treatment interval to reduce the degradation of cAMP by phosphodiesterase. As shown in Fig. 2, significant elevation of cAMP was seen after stimulation by 1 and 10 nM of α MSH in cells over-expressing MC3R or MC4R. Based on these results, the doses of 1 and 10 nM of α MSH were used for subsequent assays.

Identification of optimal dose of leptin in inducing STAT3

To identify the optimal concentration of leptin for the activation of the STAT3 signaling pathway, groups of HEK293 cells ($n=3$ for each treatment condition) were transfected with ObRb along with the STAT3-responsive luciferase reporter plasmids. The pcDNA empty vector transfected cells showed basal luciferase activity and served as a negative control. Ten and 50 nM leptin induced an almost identical STAT3 responsive luciferase activity, being significantly higher than that induced by 0.1 and 1 nM leptin ($p < 0.005$) and five-fold greater than the negative control. The STAT3 responsive luciferase activity did not exhibit any difference between the two higher doses, or the two lower doses (Fig. 3). The lowest dose (10 nM) of each ligand generating maximal downstream signaling was chosen for use in tests of signaling in MC3/4R and ObRb (+) cells.

Potentiation of leptin-induced STAT3 activation by α MSH

Groups of HEK293 cells underwent transient transfection to induce over-expression of murine ObRb along with the STAT3 luciferase reporter and basic Renilla luciferase reporter, and MC3R or MC4R. The control groups included ObRb + pcDNA3.1 (0.1 nM each), and pcDNA3.1 only (0.2 nM).

These triplicated groups of cells were treated with 10 nM of α MSH, leptin, or both (12 groups altogether) for 6 h at 18 h after transfection. The first group of bars in Fig. 4a shows the presence of basal STAT3 luciferase activity in pcDNA empty vector transfected cells. This was not affected by the addition of either α MSH or leptin. In cells over-expressing ObRb (second group of bars), leptin, but not α MSH, induced a significant increase of STAT3 activity ($p < 0.005$), and there was no additional effect of α MSH co-treatment.

In cells co-expressing MC3R and ObRb (third group of bars), α MSH alone induced a significant increase of STAT3 activity in comparison with the group of cells over-expressing only ObRb ($p < 0.005$). The enhanced STAT3 activity in the additional presence of the MC3R plasmid was not significant when the cells were treated with only leptin. Co-treatment of these cells with both α MSH and leptin, however, caused a prominent increase of STAT3, greater than that expected for either of the single treatments ($p < 0.005$ compared with single treatment of MC3R/ObRb co-expressing cells, or with the ObRb expressing cells treated with both ligands). This illustrates synergy between α MSH and leptin in STAT3 activation. In cells over-expressing both MC4R and ObRb, α MSH alone caused no significant increase of STAT3 activity, and the effect of leptin was similar to that observed in cells expressing ObRb only. However, the potentiating effect of α MSH on leptin-induced STAT3 activation was again conspicuous ($p < 0.005$ vs. only MSH or leptin treatment).

Overall, α MSH and leptin co-treatment of the HEK293 cells over-expressing ObRb along with MC3R or MC4R resulted in an almost five-fold increase of STAT3 activity. Because the cells exhibited maximal responses to both α MSH and leptin at the concentration of 10 nM, as shown

previously in Figs 2 and 3, the results shown particularly with over-expression of MC3R and ObRb indicate a synergistic effect of α MSH and leptin in activating STAT3.

Additional evidence of an interaction between α MSH and leptin was seen with the use of different doses of each. In the experiments shown in Fig. 4b, 1 nM of α MSH and 50 nM of leptin were applied to the HEK 293 cells 18 h after co-transfection with MC3R or MC4R and ObRb, with the cellular conditions otherwise being identical. The results were similar to the previous experiment involving the 10 nM dose of both ligands; co-treatment with a smaller dose (1 nM) of α MSH and a larger dose (50 nM) of leptin again induced an elevation of STAT3 in doubly transfected cells that was greater than that seen with either α MSH or leptin alone. This was particularly remarkable in MC3R/ObRb over-expressing cells ($p < 0.005$, third group of bars), and it was also significant ($p < 0.01$, fourth group of bars) in the MC4R/ObRb cells. The presence of MC3R or MC4R was essential for such effects to occur, as the cells over-expressing only ObRb (second group of bars) did not show a greater increase of STAT3 after co-treatment than was seen with only leptin.

In RBE4, cerebral microvessel endothelial cells, which show low mRNA levels of MC3R, MC4R, and ObRb, co-treatment with either α MSH or leptin did not affect STAT3 activation (first set of bars in Fig. 4c). When these cells were transfected with MC3R or MC4R (second and third set of bars), the slight increase of STAT3 production in response to leptin was not significant, but the cellular response to α MSH was more robust (6.7-fold for MC3R over-expressing cells and 4.1-fold for MC4R over-expressing cells, $p < 0.005$ for both). When the cells were transfected with ObRb (fourth set of bars), leptin treatment induced a 4.5-fold increase of STAT3 ($p < 0.005$), but α MSH had no effect. In none of the single-receptor over-expressing groups was an additive effect of co-treatment with α MSH and leptin observed.

In RBE4, cells over-expressing both MC3R and ObRb (fifth set of bars), the α MSH-induced STAT3 activation was similar to that seen in the cells over-expressing only MC3R but was significantly higher than that in the cells over-expressing only ObRb ($p < 0.005$). The effect of leptin was similar to that in ObRb-expressing cells, and significantly ($p < 0.005$) higher than that in cells over-expressing MC3R or MC4R. α MSH showed a trend toward being more effective than leptin ($p = 0.06$) in these doubly expressing cells. Combined treatment with both α MSH and leptin induced a further increase ($p < 0.005$) that appeared additive.

In RBE4, cells over-expressing both MC4R and ObRb (last group of bars), leptin treatment caused a significant ($p < 0.005$) increase of STAT3 in comparison with those over-expressing pcDNA, MC3R, MC4R, or ObRb. α MSH did not show an enhancing effect in comparison with the groups over-expressing one receptor, but combined treatment with α MSH and leptin caused a greater increase of STAT3 than treatment with either α MSH or leptin ($p < 0.005$, Fig. 4c).

α MSH potentiates leptin-induced STAT3 activation via MAPK

HEK293 cells were transfected with MC4R and ObRb expression vectors along with STAT3 responsive luciferase reporter and basic renilla luciferase reporter plasmid, and stimulated with the ligands 18 h later. Pre-treatment of the transfected cells with DMSO for 1 h before ligand stimulation did not affect the amplitudes of STAT3 activation induced by α MSH, leptin, or both observed in Fig. 4a. However, 50 μ M PD98059, the specific MEK inhibitor that blocks the MAPK signaling pathway, significantly attenuated α MSH-induced STAT3 activation as compared with the group pre-treated with the DMSO diluent ($p < 0.01$, Fig. 5). The specificity of PD98059 was shown by its lack of inhibition of leptin-induced STAT3 activation. Moreover, the presence of the MEK inhibitor dramatically reduced STAT3 activation induced by the combination of α MSH and leptin. The level of STAT3 activity was significantly lower than that observed in the DMSO-treated group ($p < 0.001$), and also lower than leptin-induced

STAT3 activity in the inhibitor-treated group ($p < 0.01$). Thus, the α MSH-potentiated leptin-induced STAT3 activation was abolished by blockade of the MAPK pathway.

Failure of leptin to potentiate α MSH-induced cAMP elevation

To determine whether the interactions between leptin and α MSH on cellular signaling are reciprocal, we further tested cAMP production in cells over-expressing ObRb and treated with leptin. As expected, α MSH (10 nM) increased cAMP in HEK293 cells over-expressing either MC3R ($p < 0.05$) or MC4R ($p < 0.005$) as compared with the group transfected with pcDNA. Leptin (10 nM) alone or in combination with α MSH did not exert an additional effect. Nonetheless, in HEK cells co-transfected with both MC3R and ObRb (last of fifth set of bars), treatment with both α MSH and leptin showed significantly greater cAMP production than the cells over-expressing MC3R only (last of second set of bars; $p < 0.005$) (Fig. 6).

Effects of MC3R or MC4R co-expression on ObRb mRNA expression

There were differential effects of MC3R and MC4R on leptin-induced STAT3 luciferase activity. In response to leptin in RBE4 cells, MC3R co-expression tended to reduce STAT3 luciferase activity ($p = 0.06$) while MC4R co-expression increased STAT3 luciferase activity ($p < 0.05$) (striped bars in Fig. 4c above). The HEK293 cells appeared to show the same pattern but the changes were not statistically significant (striped bars in Fig. 4a and b above). To test whether the level of ObRb expression was differentially altered by MC3R or MC4R, qPCR for ObRb mRNA was performed after the cells were transfected with MC3R + ObRb and MC4R + ObRb. These results were compared with those from cells transfected with pcDNA empty vector or ObRb, the amount of pcDNA being equal to that of MC3R or MC4R. In HEK293 cells, there was no significant difference in the level of ObRb mRNA in the groups over-expressing both ObRa and one of the melanocortin receptors. All were higher than the pcDNA negative control (Fig. 7a). In RBE4 cells, co-expression of MC3R did not affect the level of expression of ObRb mRNA, but co-expression of MC4R induced a significant increase of ObRb mRNA. All levels were higher than that in the pcDNA group (Fig. 7b).

Effects of MC3R or MC4R co-expression on leptin-induced STAT3 signaling

In the basal state, pSTAT3 activation in response to leptin in HEK293 cells was present at the Ser⁷²⁷ residue but minimal at the Tyr⁷⁰⁵ residue. When ObRb was over-expressed, leptin induced a major increase of pSTAT3-Y⁷⁰⁵ without affecting the baseline of S⁷²⁷. When MC3R was co-expressed with ObRb, pSTAT3 activity at both phosphorylation sites was decreased. By contrast, MC4R co-expression did not affect ObRb-mediated pSTAT3 activity in response to leptin (Fig. 8).

Discussion

To determine the crosstalk of intracellular signaling by STATs and cAMP, we first conducted dose—response studies to identify the optimal doses of leptin and α MSH. Leptin, α MSH, or both were then applied to two cellular systems: HEK293 cells that reflect peripheral tissue in which blood-borne leptin and α MSH are abundant, and RBE4 cells that represent conditions at the BBB. In HEK293 cells co-transfected with MC3R and ObRb that were co-treated with the optimal concentrations of leptin and α MSH, there was a synergistic activation of STAT3. In RBE4 cerebral endothelial cells, the STAT3 activating effect of α MSH and leptin in the doubly transfected cells was also greater than the effect of either alone.

In both HEK293 and RBE4 cells over-expressing melanocortin receptors and ObRb, α MSH potentiated leptin-induced signaling. This, however, was not reciprocal, as leptin did not potentiate α MSH-induced cAMP elevation in cells co-expressing either MC3R + ObRb or MC4R + ObRb. This phenomenon, combined with our findings that MC3R/MC4R activation

by α MSH led to substantial enhancement of leptin-induced STAT3 activity, is consistent with our previous observation of the interaction between the two receptors shared by urocortin and corticotropin-releasing hormone — CRHR1 and CRHR2 — and the leptin receptor (Pan *et al.* 2007). In that study, we showed that urocortin potentiates leptin-induced STATs signaling by either CRHR1 or CRHR2, but co-expression of ObRb does not change cAMP production or urocortin binding. The similarity between the melanocortin receptor system and CRH receptor system in their potentiation of leptin/ObRb signaling might suggest a generalized effect of GPCRs involved in anorexia. The functional significance of these novel phenomena may implicate the “partial rescue” of leptin-induced STAT3 signaling by melanocortin under conditions associated with elevated blood concentrations of leptin and signaling resistance, as seen in neonatal development or various forms of obesity (Pan and Kastin 2007b; Pan *et al.* 2008a,b).

It is known that both ObRb and MC4R can activate MAPK, p42 (ERK2), and p44 (ERK1) upon stimulation by leptin and α MSH, respectively (Björbæk *et al.* 1997; Banks *et al.* 2000; Vongs *et al.* 2004; Sutton *et al.* 2005; Patten *et al.* 2007). It has also been shown that MAPK is involved in STAT3 activation, with induction of serine phosphorylation (David *et al.* 1995; Zhang *et al.* 1995; Winston and Hunter 1996; Kuroki and O’Flaherty 1999; Stepkowski *et al.* 2008). Thus, having observed the α MSH-mediated potentiation of leptin-induced STAT3 activation, we further hypothesized that α MSH/MC4R signaling and leptin/ObRb signaling may interact and/or converge on the activation of MAPK, which in turn boosts downstream STAT3 activity. In cells over-expressing MC4R and ObRb, the specific MEK inhibitor PD98095 reduced α MSH-induced STAT3 activation to less than half that observed in the vehicle-treated group. This suggests that ligand-stimulated MC4R activates STAT3 through the MAPK pathway, although to a lesser extent than leptin-induced STAT3 activation. The ineffectiveness of the MEK inhibitor on leptin-induced STAT3 activation is consistent with the main use by leptin of the JAK-STAT pathway to activate this transcription factor. More importantly, the dramatic reduction by the MEK inhibitor of STAT3 activated by co-treatment with α MSH and leptin strongly suggests an interaction between MAPK and the leptin-induced JAK-STAT pathway. This indicates that when both ligands are available to the cells expressing MC4R and ObRb, both the α MSH-mediated potentiation and leptin signaling itself require a functional MAPK pathway to induce STAT3 activation.

In MC3R and ObRb co-expressing cells, the level of ObRb mRNA was unchanged in comparison with the cells over-expressing ObRb only (after co-transfection of pcDNA and ObRb plasmids). Although this was seen in both HEK293 cells and RBE4 cells, the effect of MC4R co-expression showed a dramatic difference between the two cell lines. MC4R caused a significant increase of ObRb mRNA only in RBE4 cells, as seen in Fig. 7. This difference might be related to the relatively high endogenous level of expression of MC4R in HEK293 cells shown in Fig. 1. In none of these groups did MC4R induce an increase of ObRb-mediated pSTAT3 activity in HEK293 cells in response to leptin.

The greater effect of leptin on pSTAT3-Y⁷⁰⁵ than pSTAT3-S⁷²⁷ is consistent with our previous observation with SHSY5Y neuronal cells (He *et al.* 2009). MC3R even caused a reduction of pSTAT3. This further suggests that α MSH-mediated potentiation of leptin signaling probably took place within the nucleus. Most likely, α MSH activates MAPK downstream to MC3R and MC4R, and induces nuclear translocation of signaling molecules such as MEKKs, JNK, and Elk1, as seen in other GPCRs (Blaukat *et al.* 1999; Dikic and Blaukat 1999). This in turn enhances STAT3 transcriptional activity by activation of the promoter. Along with a greater level of expression of ObRb, the RBE4 cells treated with both MC4R and ObRb showed greater potentiation of STAT3 signaling.

In summary, ObRb-mediated STAT3 signaling by leptin can be potentiated by α MSH in the presence of melanocortin receptors. This is partially mediated by direct activation of ObRb by α MSH, and mainly involves activation of MAPK induced by the two different types of receptors. MC4R co-expression also facilitated ObRb expression in RBE4 cells, an effect not seen with MC3R. Given the presence of cells expressing both types of receptors, the biological consequences of such interactions may not be restricted to their known involvement in obesity.

Acknowledgments

Grant support was provided by NIH (DK54880, NS45751, NS46528, and NS62291).

We thank Dr Pierre Olivier Couraud (Institut Cochin, Paris) for providing the RBE4 cells, Dr Christian Bjorbaek (Harvard Medical School) for the ObRb plasmid, and Ms. Ling Ying for technical assistance.

Abbreviations used

BBB, blood—brain barrier
 CRHR, corticotropin-releasing hormone receptor
 CSF, cerebrospinal fluid
 DMSO, dimethylsulfoxide
 GPCR, G protein-coupled receptor
 IBMX, 3-isobutyl-1-methyl-xanthine
 MAPK, mitogen-activated kinase
 MC3/4R, melanocortin 3 and 4 receptors
 MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase
 MSH, α -melanocyte stimulating hormone
 ObR, leptin receptor
 STAT3, signal transducers and activators of transcription-3.

References

- Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM. Leptin enters the brain by a saturable system independent of insulin. *Peptides* 1996;17:305–311. [PubMed: 8801538]
- Banks AS, Davis SM, Bates SH, Myers MG Jr. Activation of downstream signals by the long form of the leptin receptor. *J. Biol. Chem* 2000;275:14563–14572. [PubMed: 10799542]
- Baumann H, Morella KK, White DW, Dembski M, Bailon PS, Kim H, Lai CF, Tartaglia LA. The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc. Natl. Acad. Sci. USA* 1996;93:8374–8378. [PubMed: 8710878]
- Bjorbaek C, Kahn BB. Leptin signaling in the central nervous system and the periphery. *Recent Prog. Horm. Res* 2004;59:305–331. [PubMed: 14749508]
- Bjorbaek C, Uotani S, da Silva B, Flier JS. Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J. Biol. Chem* 1997;272:32686–32695. [PubMed: 9405487]
- Blaukat A, Pizard A, Rajerison RM, Alhenc-Gelas F, Muller-Esterl W, Dikic I. Activation of mitogen-activated protein kinase by the bradykinin B2 receptor is independent of receptor phosphorylation and phosphorylation-triggered internalization. *FEBS Lett* 1999;451:337–341. [PubMed: 10371216]
- Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 1995;269:546–549. [PubMed: 7624778]
- Coll AP, Farooqi IS, Challis BG, Yeo GS, O'Rahilly S. Proopiomelanocortin and energy balance: insights from human and murine genetics. *J. Clin. Endocrinol. Metab* 2004;89:2557–2562. [PubMed: 15181023]
- David M, Petricoin E III, Benjamin C, Pine R, Weber MJ, Larner AC. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. *Science* 1995;269:1721–1723. [PubMed: 7569900]

- Dikic I, Blaukat A. Protein tyrosine kinase-mediated pathways in G protein-coupled receptor signaling. *Cell Biochem. Biophys* 1999;30:369–387. [PubMed: 10403057]
- Fruhbeck G. Intracellular signalling pathways activated by leptin. *Biochem. J* 2006;393:7–20. [PubMed: 16336196]
- Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC. Defective STAT signaling by the leptin receptor in diabetic mice. *Proc. Natl. Acad. Sci. USA* 1996;93:6231–6235. [PubMed: 8692797]
- He Y, Kastin AJ, Hsueh H, Pan W. The Cdk5/p35 Kinases Modulate Leptin-Induced STAT3 Signaling. *J. Mol. Neurosci.* 2009in press, doi: 10.1007/s12031-008-9174-3
- Hileman SM, Pierroz DD, Masuzaki H, Bjorbaek C, El Haschimi K, Banks WA, Flier JS. Characterization of short isoforms of the leptin receptor in rat cerebral microvessels and of brain uptake of leptin in mouse models of obesity. *Endocrinology* 2002;143:775–783. [PubMed: 11861497]
- Kastin AJ, Pan W. Editorial: Intranasal leptin: blood-brain barrier bypass (BBBB) for obesity? *Endocrinology* 2006;147:2086–2087. [PubMed: 16617152]
- Kastin AJ, Akerstrom V, Pan W. Activation of urocortin transport into brain by leptin. *Peptides* 2000;21:1811–1817. [PubMed: 11150641]
- Kastin AJ, Pan W, Akerstrom V, Hackler L, Wang CF, Kotz CM. Novel peptide-peptide cooperation may transform feeding behavior. *Peptides* 2002;23:2189–2196. [PubMed: 12535698]
- Kuroki M, O'Flaherty JT. Extracellular signal-regulated protein kinase (ERK)-dependent and ERK-independent pathways target STAT3 on serine-727 in human neutrophils stimulated by chemotactic factors and cytokines. *Biochem. J* 1999;341(Pt 3):691–696. [PubMed: 10417333]
- Lee EJ, Lee SH, Jung JW, Lee W, Kim BJ, Park KW, Lim SK, Yoon CJ, Baik JH. Differential regulation of cAMP-mediated gene transcription and ligand selectivity by MC3R and MC4R melanocortin receptors. *Eur. J. Biochem* 2001;268:582–591. [PubMed: 11168397]
- Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. *Science* 1992;257:1248–1251. [PubMed: 1325670]
- Murakami T, Shima K. Cloning of rat obese cDNA and its expression in obese rats. *Biochem. Biophys. Res. Commun* 1995;209:944–952. [PubMed: 7733988]
- Myers MG Jr. Leptin receptor signaling and the regulation of mammalian physiology. *Recent Prog. Horm. Res* 2004;59:287–304. [PubMed: 14749507]
- Pan W, Kastin AJ. Adipokines and the blood-brain barrier. *Peptides* 2007a;28:1317–1330. [PubMed: 17540480]
- Pan W, Kastin AJ. Mahogany, blood-brain barrier, and fat mass surge in A^{Vy} mice. *International Journal of Obesity* 2007b;31:1030–1032. [PubMed: 17224930]
- Pan W, Akerstrom V, Zhang J, Pejovic V, Kastin AJ. Modulation of feeding-related peptide/protein signals by the blood-brain barrier. *J. Neurochem* 2004;90:455–461. [PubMed: 15228601]
- Pan W, Ding Y, Yu Y, Ohtaki H, Nakamachi T, Kastin AJ. Stroke upregulates TNF alpha transport across the blood-brain barrier. *Exp. Neurol* 2006;198:222–233. [PubMed: 16412421]
- Pan W, Tu H, Hsueh H, Daniel J, Kastin AJ. Unexpected amplification of leptin-induced Stat3 signaling by urocortin: implications for obesity. *J. Mol. Neurosci* 2007;33:232–238. [PubMed: 17952632]
- Pan W, Hsueh H, He Y, Sakharkar A, Cain C, Yu C, Kastin AJ. Astrocyte Leptin Receptor (ObR) and Leptin Transport in Adult-Onset Obese Mice. *Endocrinology* 2008a;149:2798–2806. [PubMed: 18292187]
- Pan W, Hsueh H, Tu H, Kastin AJ. Developmental changes of leptin receptors in cerebral microvessels: unexpected relation to leptin transport. *Endocrinology* 2008b;149:877–885. [PubMed: 18039787]
- Patten CS, Daniels D, Suzuki A, Fluharty SJ, Yee DK. Structural and signaling requirements of the human melanocortin 4 receptor for MAP kinase activation. *Regul. Pept* 2007;142:111–122. [PubMed: 17376547]
- Pritchard LE, Turnbull AV, White A. Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. *J. Endocrinol* 2002;172:411–421. [PubMed: 11874690]
- Rosenblum CI, Tota M, Cully D, et al. Functional STAT 1 and 3 signaling by the leptin receptor (OB-R); reduced expression of the rat fatty leptin receptor in transfected cells. *Endocrinology* 1996;137:5178–5181. [PubMed: 8895396]

- Roux F, Couraud PO. Rat brain endothelial cell lines for the study of blood-brain barrier permeability and transport functions. *Cell. Mol. Neurobiol* 2005;25:41–58. [PubMed: 15962508]
- Roux F, Durieu-Trautmann O, Chaverot N, Claire M, Mailly P, Bourre JM, Strosberg AD, Couraud PO. Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. *J. Cell. Physiol* 1994;159:101–113. [PubMed: 7908023]
- Shimizu H, Inoue K, Mori M. The leptin-dependent and -independent melanocortin signaling system: regulation of feeding and energy expenditure. *J. Endocrinol* 2007;193:1–9. [PubMed: 17400797]
- Stepkowski SM, Chen W, Ross JA, Nagy ZS, Kirken RA. STAT3: an important regulator of multiple cytokine functions. *Transplantation* 2008;85:1372–1377. [PubMed: 18497672]
- Sutton GM, Duos B, Patterson LM, Berthoud HR. Melanocortinergic modulation of cholecystokinin-induced suppression of feeding through extracellular signal-regulated kinase signaling in rat solitary nucleus. *Endocrinology* 2005;146:3739–3747. [PubMed: 15961554]
- Tu H, Kastin AJ, Bjorbaek C, Pan W. Urocortin trafficking in cerebral microvessel endothelial cells. *J. Mol. Neurosci* 2007a;31:171–182. [PubMed: 17478891]
- Tu H, Kastin AJ, Pan W. CRH-R1 and CRH-R2 are both trafficking and signaling receptors for urocortin. *Mol. Endocrinol* 2007b;21:700–711. [PubMed: 17170072]
- Tu H, Pan W, Feucht L, Kastin AJ. Convergent trafficking pattern of leptin after endocytosis mediated by ObRa — ObRd. *J. Cell. Physiol* 2007c;212:215–222. [PubMed: 17323382]
- Vongs A, Lynn NM, Rosenblum CI. Activation of MAP kinase by MC4-R through PI3 kinase. *Regul. Pept* 2004;120:113–118. [PubMed: 15177928]
- Winston LA, Hunter T. Intracellular signalling: putting JAKs on the kinase MAP. *Curr. Biol* 1996;6:668–671. [PubMed: 8793290]
- Yu Y, Kastin AJ, Pan W. Reciprocal interactions of insulin and IGF-1 in receptor-mediated transport across the BBB. *Endocrinol* 2006;147:2611–2615.
- Yu C, Kastin AJ, Ding Y, Pan W. Gamma glutamyl transpeptidase is a dynamic indicator of endothelial response to stroke. *Exp. Neurol* 2007;203:116–122. [PubMed: 16973162]
- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372:425–432. [PubMed: 7984236]
- Zhang X, Blenis J, Li HC, Schindler C, Chen-Kiang S. Requirement of serine phosphorylation for formation of STAT-promoter complexes. *Science* 1995;267:1990–1994. [PubMed: 7701321]
- Zlokovic BV, Jovanovic S, Miao W, Samara S, Verma S, Farrell CL. Differential regulation of leptin transport by the choroid plexus and blood-brain barrier and high affinity transport systems for entry into hypothalamus and across the blood-cerebrospinal fluid barrier. *Endocrinology* 2000;141:1434–1441. [PubMed: 10746647]

Fig. 1.

The presence of MC3R and MC4R mRNA was shown in mouse cerebral capillaries (EnC), RBE4 endothelia, and HEK293 cells by RT-PCR. (-) negative controls minus reverse transcriptase. (+) positive controls in which mouse MC3R and MC4R plasmids were used as the templates.

Fig. 2. cAMP production by α MSH in HEK293 cells over-expressing MC3R or MC4R ($n = 3$ /group). In each group of bars, significant differences from the pcDNA-transfected group are shown by asterisks above the bar. *** $p < 0.005$.

Fig. 3. HEK293 cells over-expressing ObRb or mock-transfected with pcDNA were treated with 0.1, 1, 5, or 50 nM of leptin ($n = 3/\text{group}$). Significant elevation of STAT3 luciferase activity was seen at 5–50 nM of leptin. *** $p < 0.005$ compared with the 0 and 0.1 nM doses.

Fig. 4.

STAT3 activation measured by luciferase reporter assay ($n = 3/\text{group}$). $+p = 0.06$; $*p < 0.05$; $**p < 0.01$; $***p < 0.005$. (a) In HEK293 cells, co-treatment with the optimal doses (10 nM) of leptin and αMSH induced a significant increase of STAT3 activity in ObRb + MC3R or ObRb + MC4R cells, greater than that seen by treatment with either single ligand. (b) In HEK293 cells, co-treatment with a smaller dose (1 nM) of αMSH and a larger dose (50 nM) of leptin induced a significant increase of STAT3 activity in ObRb + MC3R or ObRb + MC4R cells, greater than that seen by treatment with either single ligand. (c) In RBE4 cerebral endothelial cells, co-treatment with the optimal doses (10 nM) of leptin and αMSH induced a

significant increase of STAT3 activity in ObRb + MC3R and ObRb + MC4R cells, greater than that seen by treatment with either single ligand.

Fig. 5.

HEK cells over-expressing both MC4R and ObRb were pre-treated with 50 μ M PD98059 and the diluent (0.1% DMSO), followed by treatment of optimal doses (10 nM) of either α MSH, leptin, or both ($n = 3$ /group). PD98059 significantly reduced STAT3 activation induced by α MSH alone, and by co-treatment with both α MSH and leptin, but had no effect on leptin-induced STAT3 activity. ** $p < 0.01$, *** $p < 0.001$ as compared with the respective DMSO controls.

Fig. 6.

cAMP production in singly and doubly transfected cells after treatment with α MSH, leptin, or both ligands ($n = 3/\text{group}$). By contrast with the robust effect of α MSH in each group of cells, leptin was without effect. However, in cells co-transfected with MC3R and ObRb, the effect of co-treatment with α MSH and leptin was significantly greater than the effect of the same co-treatment in cells transfected only with MC3R, suggesting an ability of leptin to increase cAMP production in the presence of α MSH in cells doubly transfected. Cells transfected only with ObRb released no more cAMP than the empty vector controls in response to α MSH, leptin, or both.

Fig. 7.

Effects of MC3R or MC4R co-transfection on the expression of ObRb mRNA. *** $p < 0.005$ in comparison with the pcDNA control ($n = 3/\text{group}$). (a) In HEK293 cells, MC3R or MC4R co-expression did not significantly affect the level of ObRb mRNA 24 h after transfection. (b) In RBE4 cells, MC4R co-expression significantly ($p < 0.005$) increased the level of ObRb mRNA, whereas MC3R co-expression did not have a significant effect.

Fig. 8.

Effects of MC3R or MC4R co-expression on ObRb-mediated pSTAT3 level after leptin stimulation (30 nM for 1 h). There was a decrease of pSTAT3-Y⁷⁰⁵ and pSTAT3-S⁷²⁷ in MC3R co-expressing cells. There was no apparent change in cells co-expressing MC4R. The housekeeping gene β -actin was unchanged by transfection or cell treatment.

Table 1

PCR primers for mRNA detection in biological samples

Gene	Species	Forward	Reverse
MC3R	Mouse	5'-CTGCTGCCTGTCTTCTGTTCT	5'-AGAGAATCTCCTTGAACGTGTG
MC4R	Mouse	5'-ATGGCATGTATACTTCCCTCCA	5'-CCTCCCAGAGGATAGAAACAGA
MC3R	Rat	5'-CTTATCCGACGCTGCCTAAC	5'-ACCGCAGAGAATCTCCTTGA
MC4R	Rat	5'-CTTCCCTCCACCTCTGGAAC	5'-CCCAGGGGGTAGAAACAGTA

Table 2

Cloning primers for mouse MC3R and MC4R

Plasmid	Forward	Reverse
MC3R	5'-GCGCTAGCCACCATGAACTCTTCCTGCTGCCTGT	5'-GCGGATCCCTAGCCCAAGTTCATGCTGT
MC4R	5'-GCGCTAGCCACCATGAACTCCACCCACCACCATG	5'-GCGGATCCTTAATACCTGCTAGACAACT

Underlined nucleotides represent Kozak sequences.

Table 3

ObRb and GAPDH primers and fluorescent probes for qPCR

Gene	Forward primer (FP) and reverse primer (RP)	Probes
ObRb (mouse cDNA)	FP: GCATGCAGAATCAGTGATATTTGG RP: CAAGCTGTATCGACACTGATTTCTTC	6FAM-CCTCTTCTTCTGGAGCCTGAACCCATTTT-TAMRA
GAPDH (for HEK293)	FP: CCAGGTGGTCTCCTCTGACTTC RP: GTGGTCGTTGAGGGCAATG	6-FAM-CACCCACTCCTCCACCTTTGACGCT-TAMRA
GAPDH (for RBE4)	FP: TGTGTCCGTCGTGGATCTGA RP: CCTGCTTCACCACCTTCTTGA	6-FAM-CCGCCTGGAGAAACCTGCCAAGTATG-TAMRA