Melanocortins induce interleukin 6 gene expression and secretion through melanocortin receptors 2 and 5 in 3T3-L1 adipocytes

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Abstract

Interleukin 6 (IL6) is a pleiotropic cytokine that not only affects the immune system, but also plays an active role in many physiological events in various organs. Notably, 35% of systemic IL6 originates from adipose tissues under noninflammatory conditions. Here, we describe a previously unknown function of melanocortins in regulating IL6 gene expression and production in 3T3-L1 adipocytes through membrane receptors which are called melanocortin receptors (MCRs). Of the five MCRs that have been cloned, MC2R and MC5R are expressed during adipocyte differentiation. α-Melanocyte-stimulating hormone (α-MSH) or ACTH treatment of 3T3-L1 adipocytes induces IL6 gene expression and production in a time- and concentration-dependent manner via various signaling pathways including the protein kinase A, p38 mitogen-activated protein kinase, cJun N-terminal kinase, and IκB kinase pathways. Specific inhibition of MC2R and MC5R expression with short interfering Mc2r and Mc5r RNAs significantly attenuated the α-MSH-induced increase of intracellular cAMP and both the level of IL6 mRNA and secretion of IL6 in 3T3-L1 adipocytes. Finally, when injected into mouse tail vein, α-MSH dramatically increased the IL6 transcript levels in epididymal fat pads. These results suggest that α-MSH in addition to ACTH may function as a regulator of inflammation by regulating cytokine production.

Journal of Molecular Endocrinology (2010) 44, 225–236

Introduction

Interleukin 6 (IL6) is a pleiotropic cytokine involved in the regulation of the immune response, acute-phase reaction, nervous and endocrine system function, bone metabolism, hematopoiesis, insulin resistance, and various human diseases (Kamimura et al. 2005). Notably, adipose tissue has been regarded as a major source of circulating IL6, particularly in obesity, because 35% of systemic IL6 originates from subcutaneous adipocytes (Mohamed-Ali et al. 1997, Fried et al. 1998). Recent studies have demonstrated that IL6 negatively affects insulin signaling in adipocytes by reducing expression of insulin receptor signaling components (Rotter et al. 2003), by inducing the suppressor of cytokine signaling 3 (a negative regulator of insulin signaling; Lagathu et al. 2003, Shi et al. 2004), and by decreasing adiponectin secretion (Fasahauer et al. 2003, Kristiansen & Mandrup-Poulsen 2005). Many IL6 inducers are known including IL1β, tumor necrosis factor-α, lysophosphatidic acid, prostaglandin E, thyroid-stimulating hormone, platelet-derived growth factor, catecholamine, palmitate, bacterial lipopolysaccharide (LPS), and viral infection and associated transforming growth factor-β (Verhasselt et al. 1997, Franchimont et al. 1999, Legrand-Poels et al. 2000, Fang et al. 2004, Ajuwon & Spurlock 2005, Liu et al. 2005, Tan et al. 2007, Antunes et al. 2008, Diya et al. 2008). This is the first time that melanocortins induce IL6 gene expression and production through the MCRs.

Melanocortins are generated by the proteolytic cleavage of the precursor molecule, proopiomelanocortin (POMC). Differential enzymatic cleavage of POMC by prohormone convertases results in the production of ACTH, α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH, and other hormones. Even though these hormones are derived from different regions of the precursor POMC, because they share a core binding sequence His-Phe-Arg-Trp (HFRW), which is required for binding and activation of the receptors, ACTH can activate all five MCRs, whereas α-MSH can activate all MCRs except MC2R (Yang & Harmon 2003). α-MSH is one of the POMC gene products.
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Materials and methods

3T3-L1 cell cultures

3T3-L1 cells were grown in DMEM containing 1% penicillin/streptomycin (P/S) and 10% fetal bovine serum (FBS) until they reach confluency. They were then differentiated with the differentiation mixture (DMEM containing 1% P/S and 10% FBS, 500 μM 3-isobutyl-1-methylxanthine, 10 μg/ml insulin, and 1 μM dexamethasone) for 2 days as described (Jun et al. 2006). Insulin was present for an additional 4 days, and then, the culture medium was changed with only DMEM (1% P/S and 10% FBS) for 2 days. Each medium was changed every 2 days.

RNA extraction and real-time quantitative reverse transcription-PCR

Total RNA was extracted from differentiated adipocytes using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. For real-time quantitative reverse transcription (RT)-PCR, total RNA was reversely transcribed using an ImProm-II RT system (Promega) according to the manufacturer’s instructions. For detection and quantification, a MyiQ real-time PCR detection system (Bio-Rad) was used. PCRs were performed using a SYBR Premix Ex Taq II (Takara, Seoul, Republic of Korea). PCRs were carried out in a final volume of 20 μl using 0.5 μM of each primer, cDNA, and 10 μl of the supplied enzyme mixture containing the DNA double-strand-specific SYBR Green I dye for detection of PCR products. PCRs were performed with a 3-min pre-incubation at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. PCR products were verified by melting curve analysis, agarose gel electrophoresis, and DNA sequencing.

The following primers were used:

\[ \text{Arbp} \text{(forward, 5'-AAAACCTCGGATCTGGATTTAATTTAG; reverse, 5'-TAATTCACAAGCTGAAAATCTTTGT),} \]
\[ \text{Gapdh} \text{(forward, 5'-GCCATCAATGACCCCTTCATT; reverse, 5'-GGTCTGAGATGTTGATG),} \]
\[ \text{Add1} \text{(forward, 5'-CATCTGTGTGAAGGTTATTTGCTG; reverse, 5'-AGATGACTAGGGAACGTGTGTGGT),} \]
\[ \text{PPARγ} \text{(forward, 5'-TTGCTAGACGACGCAGCCCATG-GAG; reverse, 5'-GTCTCCTGAGATCTCCTGGAGCAG),} \]
\[ \text{C/EBPα} \text{(forward, 5'-CCATTATTTTGCTTTTTG-TTGG; reverse, 5'-CTACATACACCCTTGGACACATG),} \]
\[ \text{Pref1} \text{(forward, 5'-CATGCAAAGCGCTCAACAGAGTAC; reverse, 5'-GTTATATCTGCAACAGAGGCTCTTTC),} \]
\[ \text{Pael} \text{(forward, 5'-CTGGTGCAACCACCTTTGATTAA-GATA; reverse, 5'-AAATCAGAGAGAAGAGGAGGAGAG),} \]

The authors also describe the role of melanocortins in stress-mediated response to stress. It has been postulated that stress may be a causal factor in metabolic syndromes such as insulin resistance, type 2 diabetes, and hypertension via perturbation of the HPA axis (Gohil et al. 2001, Kaufman et al. 2007). The pituitary gland secretes critical hormones which affect many target tissues in our body. On the other hand, adipocyte tissue expresses receptors for many hormones including pituitary hormones, and secretes several factors which are called adipotropins (Schaffler et al. 2006). So, adipocyte tissue is not a passive organ any longer, and this is called hypothalamic–pituitary–adipose axis. To date, five MCRs have been described (MC1R–MC5R). All are coupled to stimulatory G-proteins (Gs), which after binding to melanocortins initiate the activation of adenyl cyclase, ultimately resulting in the transcription of cAMP-responsive genes (Bohm et al. 2006). MC1R and MC2R have classically been considered to have a role in the skin pigmentation (Schaffler et al. 2006) and adrenal steroidogenesis respectively (Cone 2006). MC3R and MC4R are expressed primarily in the hypothalamus of the central nervous system (CNS). The best characterized effects of MC3R and MC4R are in the control of food intake, energy expenditure, and sexual function (Wikberg & Mutulis 2008), and disruption of Mc4r gene has been shown to cause obesity in mice (Butler et al. 2001). Among the five subtypes of MCRs, MC5R is a relatively ubiquitous receptor in peripheral tissues, suggesting a direct peripheral action for melanocortins. MC5R participates in the control of exocrine secretions in exocrine glands and fatty acid oxidation in skeletal muscle (Chen et al. 1997, An et al. 2007).

The melanocortin peptides ACTH, α-MSH, and β-lipotropin have long been recognized to have different degrees of lipolytic activity in the adipocytes of various mammalian species (Boston 1999). ACTH has recently been shown to inhibit leptin production in 3T3-L1 adipocytes (Norman et al. 2003) and to promote a pro-inflammatory adipokine profile (Iwen et al. 2008). However, the peripheral effects of α-MSH and MCRs in adipocytes have not been extensively investigated. In this study, we demonstrate for the first time that melanocortins are inducers of Il6 in 3T3-L1 adipocytes. We show that MC5R mediates the α-MSH-induced increase in Il6 gene expression, and that MC2R mediates the ACTH-induced increase in Il6 gene expression via the activation of the protein kinase A (PKA), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and IκB kinase (IKK) signaling pathways.
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Western blot analysis

3T3-L1 adipocytes were plated in 60-mm tissue culture dishes and treated with melanocortins for various time periods. After treatment, the cells were washed twice with cold PBS and then lysed with lysis buffer (250 mM Tris–Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0-02% bromophenol blue, and 10% glycerol). Equal amounts of whole cell lysates were resolved by 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were blocked using Tris-buffered saline with Tween 20 (TTBS, 150 mM Tris–Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue, and 10% glycerol).

The membranes were probed with HRP-conjugated secondary antibody to allow for the detection of the appropriate bands using an ECL detection system (Neuronex Co., Daegu, Republic of Korea).

Measurement of [3H] cAMP

Intracellular cAMP generation was determined by [3H] cAMP competition assay in binding to cAMP-binding protein as described previously by Jun et al. (2006) with some modifications. To determine the cAMP production induced by melanocortins, the 3T3-L1 adipocytes were stimulated with agonists for 20 min in the presence of the phosphodiesterase inhibitor Ro 20-1724 (5 μM), and the reaction was quickly terminated by three repeated cycles of freezing and thawing. The samples were then centrifuged at 12,000 g for 5 min at 4°C. The cAMP assay is based on the competition between [3H]-labeled cAMP and unlabeled cAMP present in the sample for binding to a crude cAMP-binding protein prepared from bovine adrenal cortex according to the method of Brown et al. (1971). Bound [3H] cAMP in the supernatant was then determined by liquid scintillation counting. Each sample was incubated with 50 μl [3H]-labeled cAMP (5 μCi) and 100 μl binding protein for 2 h at 4°C. Separation of protein-bound cAMP from unbound cAMP was achieved by absorption of free cAMP onto charcoal (100 μl), followed by centrifugation at 12,000 g for 4°C. The 200-μl supernatant was then placed into an Eppendorf tube containing 1.2 ml scintillation cocktail to measure radioactivity. The cAMP concentration in the sample was determined based on a standard curve and expressed as picomoles per microgram of protein.

ELISA

Medium from differentiated 3T3-L1 cells cultured in 24-well plates was analyzed for IL6 using a kit (R&D systems, Minneapolis, MN, USA).

RNA interference and transfection

The siRNA SMART pool containing 50 nmol of a mixture of four oligonucleotides which target mouse Mc2r and Mc5r mRNA destruction by RISCs was purchased from Dharmacon, Inc (Lafayette, CO, USA). Transfection of the 3T3-L1 adipocytes with the siRNA SMART pool oligonucleotides was carried out with a microporator (Digital Biotechnology, Daegu, Republic of Korea) according to the protocol provided by the company.

Animal experiment

Eight-week-old C57BL/6j male mice were obtained from the Orient Bio (Sungnam, South Korea). All the mouse experiments were performed in the animal facility.
under POSTECH institutional guidelines. The mice were kept in individual cages in a room in which lighting was controlled (12 h light:12 h darkness), and the temperature was maintained at 23 °C. Mice were sacrificed by cervical dislocation 0, 1, 3, and 6 h after injecting vehicle or 1 mg/kg of α-MSH i.v. Immediately after the mice were sacrificed, epididymal fat pads were removed. Total RNA was extracted from epididymal fat pads using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol, and then Il6 transcript levels were measured using a real-time quantitative RT-PCR.

Materials

α-, β-, and γ-MSH, SB203580, SP600125, Bay11-7085, H89, 6-Bnz-cAMP, forskolin, isoproterenol, and LPS were purchased from the Sigma–Aldrich Inc. ACTH was purchased from Phoenix Pharmaceuticals Company Inc. (Burlingame, CA, USA).

Statistical analysis

All numerical values are given as mean±s.d. Significance of differences between mean values of two groups was evaluated using Student’s t-test for unpaired data as appropriate. A probability of P<0.001 or P<0.05 was considered significant.

Results

MCR subtypes are expressed in metabolic tissues and 3T3-L1 adipocytes

To investigate the expression of the five MCR subtypes in mouse metabolic tissues and 3T3-L1 adipocytes, real-time quantitative RT-PCR was carried out using primers specific for each subtype. Both MC2R and MC5R were the two most abundant MCRs in all adipose tissues (subcutaneous, retroperitoneal, and epididymal fat), and the patterns of MCR expression in 3T3-L1

Figure 1 Functional expression of melanocortin receptor subtypes in mouse tissues and 3T3-L1 adipocytes. (A) Real-time quantitative RT-PCR analysis of melanocortin receptor subtypes in mouse metabolic tissues and 3T3-L1 adipocytes. Detection of mRNA corresponding to all five Mcrs in liver, muscle, subcutaneous fat, retroperitoneal fat, epididymal fat, and 3T3-L1 adipocytes. (B) Real-time quantitative RT-PCR analysis of Mc2r and Mc5r during adipogenesis. The cDNAs derived from the 3T3-L1 cells which had undergone differentiation for various time periods were amplified using specific primers. For comparison, five differentiation-specific genes were also analyzed: PPARγ, C/EBPa, Add1, aP2, and Pref1. The relative expression of the transcripts was normalized to acidic ribosomal phosphoprotein P0 (Arbp) mRNA levels, and values represent the mean±s.d. (C) Concentration-dependent effects of melanocortins on cAMP generation. 3T3-L1 adipocytes were treated with various concentrations of α-MSH, β-MSH, γ-MSH, or ACTH for 20 min, and then cAMP production was measured. The data present the means±s.d. of triplicate samples. Each experiment was performed independently at least three times.
adipocytes were similar to those of adipose tissues (Fig. 1A). Next, we investigated whether the levels of Mc2r and Mc5r were changed during adipocyte differentiation. Levels of individual transcripts were analyzed by real-time quantitative RT-PCR during the time course of 3T3-L1 differentiation up to 6 days post induction. The kinetics of Mcr mRNA expression were compared to those of several markers of adipogenesis, including the transcription factors peroxisome proliferator-activated receptor γ (PPARγ), CAAT/enhancer-binding protein α (C/EBPa), and adipocyte determination differentiation factor 1 (Add1), as well as adipose lipid-binding protein (aP2). We also assayed the kinetics of the expression of the preadipocyte marker, preadipocyte factor 1 (Pref1; Fig. 1B). These data indicate that adipogenesis is accompanied by the upregulation of Mc2r and Mc5r mRNAs. MCRs are G-protein-coupled receptors and are coupled to adenyl cyclase via Gs (Cone 2006). Because mRNA expression is not necessarily correlated with protein expression or activity, the capacities of the melanocortins to stimulate cAMP production were compared in order to characterize their pharmacological properties in 3T3-L1 adipocytes. As shown in Fig. 1C, ACTH was most active in elevating the level of intracellular cAMP followed by α-MSH, β-MSH, and γ-MSH. This is consistent with the properties of the MC2R subtype, which is selective for ACTH, and of the MC5R subtype, which binds preferentially to α-MSH, with equal affinity to β-MSH and ACTH, and with lowest affinity to γ-MSH (Cone 2006). So, α-MSH is the most potent activator of MC5R. These data suggest that MC2R and MC5R are functionally active in 3T3-L1 adipocytes.

**Melanocortins induce IL6 expression in 3T3-L1 adipocytes**

We examined the effect of α-MSH and ACTH on the level of adipocytokines in 3T3-L1 adipocytes. Of the adipocytokines tested, only IL6 was induced by...
treatment with α-MSH (Fig. 2A) or ACTH (Fig. 2B). Levels of Il6 mRNA were elevated within 1 h of α-MSH treatment and returned to baseline level by 3 h, as determined by real-time quantitative RT-PCR (Fig. 2A). Dramatic increase of Il6 mRNA was observed when 50 nM ACTH was administered (Fig. 2B). α-MSH at a concentration of 3–300 nM induced Il6 gene expression in a concentration-dependent manner, but it did not induce the expression of Pael (negative control; Fig. 2C). In agreement with this finding, the level of IL6 protein secreted into the culture medium of 3T3-L1 adipocytes increased steadily after α-MSH or ACTH addition, and peaked at ~3 h as determined by IL6 ELISA (Fig. 2D). ACTH increased Il6 gene expression to higher levels than seen for α-MSH, but the profile of the time course was similar to that of α-MSH. LPS was included in the assay as a positive control. No further increase in the level of IL6 was detected when the experiment was extended to 24 h (data not shown), indicating that maximal IL6 protein production was attained by 3 h. Taken together, these data suggest that melanocortins function as inducers of Il6 expression in 3T3-L1 adipocytes.

PKA, p38 MAPK, JNK, and IKK signaling pathways are involved in the melanocortin-mediated increase in Il6 gene expression and secretion

It has been reported that several stimuli including proinflammatory cytokines, β2-adrenergic receptor agonists, and adiponectin induce IL6 production through the p38 MAPK, PKA, and NFκB signaling pathways in various tissues and cells (Legrand-Poels et al., 2000, Tang et al., 2007). Therefore, we investigated the possibility that these mediators are also involved in the signal transduction pathway leading to increased IL6 production in α-MSH-treated 3T3-L1 adipocytes. Pretreatment of cells with either the p38-specific inhibitor SB203580 or the IKK inhibitor Bay11-7082 for 30 min prior to α-MSH treatment attenuated α-MSH-induced Il6 production, whereas the JNK inhibitor SP600125 markedly potentiated α-MSH-induced Il6 production (Fig. 3A–C). The results suggest that p38 MAPK and IKK signaling pathways activate α-MSH-induced Il6 gene expression, but JNK signaling pathway inhibits α-MSH-induced Il6 gene expression. Western blot analysis revealed that α-MSH stimulation

![Figure 3](image-url)
α-MSH 0 2 5 10 15 30 (min)
p-JNK (T183/Y185) JNK
p-cJun (S63) cJun
p-p38 (T180/Y182) p38
p-IKKα/β (S176/180) IKKα
GAPDH

Figure 4 Phosphorylation of JNK, p38 MAPK, and IKK in response to α-MSH. Serum-starved (12 h) 3T3-L1 adipocytes were treated with α-MSH (300 nM) for the indicated time periods. Whole-cell extracts were analyzed by western blots of phospho-JNK, phospho-cJun, phospho-p38 kinases, and phospho-IKK protein. Equal loading of protein was checked by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Each experiment was performed independently at least three times.

Involvement of the MC5R in the α-MSH-induced increase in Il6 gene expression and MC2R in the ACTH-induced increase in Il6 gene expression

To investigate the role of the MC5R subtype in the α-MSH-mediated increase in Il6 production and of MC2R subtype in the ACTH-mediated increase in Il6 production, we specifically inhibited MC5R expression by RNAi using a short interfering Mc5r RNA (siMC5R) and Mc2r RNA (siMC2R) in 3T3-L1 adipocytes (Fig. 6A and C). Suppression of MC5R expression significantly attenuated the α-MSH-induced increase in Il6 mRNA level (Fig. 6B), and suppression of MC2R expression also significantly attenuated the ACTH-induced increase in Il6 mRNA level (Fig. 6D) in 3T3-L1 adipocytes. However, suppression of MC5R expression could not attenuate the ACTH-induced increase in Il6 mRNA level, and suppression of MC2R expression could not attenuate α-MSH-induced increase in Il6 mRNA level (data not shown). These results suggest that MC5R is involved in the α-MSH-induced Il6 expression, and that MC2R is involved in the ACTH-induced Il6 expression in 3T3-L1 adipocytes.

Effect of α-MSH on Il6 expression in primary fat tissue

To investigate whether α-MSH regulates Il6 expression in primary fat tissue, the level of Il6 mRNA in epididymal fat pads was measured. Mice were i.v. injected with α-MSH (1 mg/kg) in the tail vein, and Il6 mRNA level in fat pads was measured at 0, 1, 3, and 6 h after α-MSH injection. Il6 transcript level in fat pads peaked 1 h after injection and returned rapidly to the baseline by 6 h (Fig. 7), which correlated well with the profile of α-MSH-induced Il6 gene expression in 3T3-L1 adipocytes. These results suggest that MCRs function to trigger Il6 expression in response to α-MSH in fat tissue.

Discussion

In contrast to the abundant data available on the classical roles of melanocortins in processes such as skin pigmentation, steroidogenesis, energy expenditure, and appetite regulation in the CNS (Cone 2006), little is known about the peripheral functions of melanocortins, particularly in the adipose tissues and in melanocortin interaction with adipocytokines. It has been reported that adipose tissues contribute as much as 35% of the basal circulating IL6. Moreover, there is a positive correlation between elevated IL6 and obesity and insulin resistance (Fernandez-Real & Ricart 2003). Here, we further characterized IL6 as a newly described protein target of the melanocortin signaling pathway in adipocytes. Recently, other groups (Hoch et al. 2008, Iwen et al. 2008) reported that melanocortin induced Il6 gene expression, which was revealed by real-time quantitative RT-PCR analysis. In this study, we have demonstrated that melanocortins increase Il6 gene expression and secretion through MCRs in 3T3-L1 adipocytes and mouse epididymal fat tissue. In addition, we have investigated knockdown effects on each MCR. These results suggest that α-MSH-induced...
increase in Il6 production is mainly mediated by MC5R, and that ACTH-induced increase in Il6 production is mediated by MC2R. PKA, p38 MAPK, JNK, and IKK signaling pathways played important roles in the melanocortin-mediated Il6 production.

We have demonstrated that the patterns of MCR expression in the 3T3-L1 adipocytes are in agreement with those of retroperitoneal, epididymal, and subcutaneous fat tissues. The differential expression profiles of five MCRs in 3T3-L1 cells have been reported (Norman et al. 2003). The expression pattern of MCRs has been analyzed with RT-PCR. We also performed RT-PCR and real-time quantitative RT-PCR. The analysis that was applied for the profile of MCRs was applied to mouse tissues. We found and confirmed that they expressed all types of MCRs by sequencing RT-PCR products, but of the five MCRs subtypes, MC2R and MC5R are expressed at the highest level in all adipose tissues. In addition to its role in the induction of IL6, described in this study, α-MSH has a direct effect on lipid metabolism and leptin regulation (Hoggard et al. 2004, Harmer et al. 2008). The observed effects of ACTH and α-MSH on IL6 expression and secretion occur at concentrations similar to those that induce lipolysis and inhibit leptin expression in adipocytes. We have assumed that ACTH and α-MSH act primarily on the MC2R and MC5R respectively (Cone 2006), since α-MSH does not bind to MC2R and the hierarchical arrangement of melanocortins with regard to elevating intracellular cAMP level is evidenced for the functional expression of MC5R in 3T3-L1 adipocytes (Fig. 1C). Finally, the MC5R knockdown experiment confirmed the involvement of MC5R in α-MSH-induced Il6 production (Fig. 6B), and MC2R in ACTH-induced Il6 production (Fig. 6D).

Widely considered a primary regulator of inflammation, p38 integrates inflammatory responses by regulating several aspects of target gene transcription and translation. p38 enhances both the transcriptional activity of NFκB via acetylation of p65 (Saha et al. 2007) and the translation of inflammatory cytokines, including IL6, via stabilization of their mRNAs by

Figure 5 PKA, p38 MAPK, JNK, and IKK are involved in ACTH-induced Il6 gene expression. Differentiated 3T3-L1 adipocytes were pretreated with A, SB203580 (10 μM); B, SP600125 (10 μM); C, Bay 11-7085 (10 μM); and D, H89 (1 μM) for 30 min prior to treatment with ACTH (50 nM) for 1 h. Data represent the means ± S.D. of triplicate samples. *P<0.05; **P<0.001 compared with the ACTH-treated group. Each experiment was performed independently at least three times.
phosphorylation of AU-rich element-binding proteins (Zhao et al. 2008). Because MCRs are coupled to adenyl cyclase and several cAMP-elevating GPCR ligands such as prostaglandin E2, thyroid-stimulating hormone, and catecholamine have been shown to upregulate Il6 mRNA expression in different cell lines (Mohamed-Ali et al. 2001, Liu et al. 2005, Antunes et al. 2006), it is reasonable to hypothesize that melanocortins activate Il6 production in adipocytes. The cAMP signal transduction pathways can activate the exchange protein directly activated by cAMP (EPAC), which acts independently of PKA (Holz et al. 2006). However, a specific activator of EPAC (8-CPT-2′OMe-cAMP) did not induce IL6 secretion in adipocytes (data not shown), whereas a specific activator of PKA (6-Bnz-cAMP) potentiated α-MSH-induced Il6 expression (Fig. 3D). These data suggest that the PKA, but not the EPAC, pathway is involved in the melanocortin-induced Il6 production in 3T3-L1 adipocytes.

In this study, we have described a novel pro-inflammatory role for MCRs in adipocytes. IKK/NFκB activation and JNK pathway are critical regulators of inflammation, and have been reported to be necessary for IL6 induction in many cells (Libermann & Baltimore 1990, Tuyt et al. 1999, An et al. 2003). The induction of IL6 has been reported. The classical pathway of NFκB activation is controlled by the IKK complex. Activated IKK phosphorylates IkB, which is then ubiquitinated and rapidly degraded, allowing NFκB to translocate from the cytoplasm to the nucleus, where it activates gene transcription (Niederberger & Geisslinger 2008). While the activation of the MCRs expressed in immune cells has been reported to have an anti-inflammatory effect, inhibiting NFκB activation and cytokine production induced by pro-inflammatory stimuli such as LPS (Yoon et al. 2003), our results nonetheless demonstrated that the activation of MCRs in the absence of another pro-inflammatory stimulus leads to the upregulation of Il6 mRNA and protein levels in adipocytes. JNK activation is also involved in the expression of IL6 in human monocytes (Tuyt et al. 1999), but α-MSH-mediated JNK activation suppresses Il6 production in adipocytes as shown in Figs 3–5. It seems likely that adipocytes do not share the same regulatory pathways that have been identified in other cell types, such as immune cells. Moreover, recent
studies on the IL6 promoter have demonstrated that induction of IL6 by several transcription factors including C/EBPz, AP1, CREB, and NFkB occurs in a highly stimulus- or cell-specific manner (Fasshauer et al. 2003, Persson et al. 2005, Chen et al. 2006).

Since adipose tissue has recently been regarded as a fast-acting endocrine organ within the hierarchy of the hypothalamus and pituitary gland and because adipose tissue expresses specific receptors for pituitary hormones and hypothalamic releasing factors at both mRNA and protein levels (Schaffler et al. 2006), our findings provide further evidence of a significant interaction between adipokytokines and neupeptides such as z-MSH and ACTH. Our findings provide evidence for the existence of a hypothalamic–pituitary–adipose axis, and for the concept of ‘adipotropins’, which describe the roles of pituitary and hypothalamic hormones or releasing factors which directly target adipocytes via their specific receptors (Schaffler et al. 2006).

We have measured Il6 expression level after injection of 1 mg/kg of z-MSH. The concentration which we used is about 5 μM per mouse approximately based on the blood volume which is about 6–8% of body weight. In most cases, the range from 200 μg/kg to 10 mg/kg for the concentration of z-MSH is commonly used. The i.v. injection of z-MSH increased Il6 production as shown in Fig. 7. To confirm this, we administered an abdominal injection of z-MSH of 1 mg/kg, and showed similar results (data not shown). This result demonstrates that z-MSH plays an important role in inducing Il6 expression in vivo.

The physiological relevance of melanocortin-induced Il6 production in adipose tissue remains unclear. However, pituitary melanocortins appear to have stress-related effects on adipocyte function. Brain-derived z-MSH is released during stress including immobilization stress (Khorram et al. 1985), and the melanocortinergic pathway is rapidly recruited under conditions of emotional stress (Liu et al. 2007). In addition, it has been suggested that in the case of ACTH- or z-MSH-induced lipolysis in adipocytes, stress-induced lipolysis is mediated by pituitary release of melanocortins (Schaffler et al. 2006). Recently, human visceral and subcutaneous adipocytes have also been shown to express the corticotropin-releasing hormone receptor types 1 and 2, which play a major role in coordinating autonomic, cardiovascular, endocrine, and behavioral responses to stress (Seres et al. 2004). These data imply that adipose tissue may be a direct peripheral target organ of stress responses. On the other hand, recent studies have demonstrated that the production of IL6 can be directly stimulated by emotional depression and stressful experiences (Kiecolt-Glaser et al. 2005). In addition, circulating IL6 levels and secretion of IL6 from adipose tissue correlate with insulin resistance (Kristiansen & Mandrup-Poulsen 2005). In this sense, our data suggest that IL6 could be a link between obesity, stress response, and insulin resistance.

To elucidate melanocortin’s direct peripheral effects on adipocytes under conditions of stress, in vivo experiments addressing the role of melanocortin in the relationship between stress responses and insulin sensitivity are needed.

In summary, these studies provide direct evidence for peripheral action of melanocortins on Il6 expression and production in adipocytes, and suggest that melanocortins may function as regulators of inflammation by regulating cytokine production. This is the first time that we find melanocortin-mediated Il6 gene expression through adipocyte membrane receptors and signaling pathways through a knockdown experiment using siRNA and in vivo. In addition, our findings provide evidence for the existence of a regulatory loop between the HPA axis and circulating Il6.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grants (No. 20090063547 and No. 20090081464), Korea Science and Engineering Foundation (KOSEF) grants (No. M10641000077-08N141007710), Regional Core Research Program/ Anti-ageing and Well-being Research Center and Brain Korea 21 program funded by the Korean Ministry of Education, Science and Technology.
Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S & Coppack SW 1997 Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *Journal of Clinical Endocrinology and Metabolism* 82 4196–4200.

Mohamed-Ali V, Flower L, Sethi J, Hotamisligil G, Gray R, Humphries SE, York DA & Pinkney J 2001 β-Adrenergic regulation of IL-6 release from adipose tissue: in vivo and in vitro studies. *Journal of Clinical Endocrinology and Metabolism* 86 5864–5869.

Niederberger E & Geisslinger G 2008 The IKK-NF-κB pathway: a source for novel molecular drug targets in pain therapy? *FASEB Journal* 22 3432–3442.

Norman D, Isidori AM, Frajese V, Caprio M, Chew SL, Grossman AB, Clark AJ, MichaelBesser G & Fabbri A 2003 ACTH and alpha-MSH inhibit leptin expression and secretion in 3T3-L1 adipocytes: model for a central-peripheral melanocortin-leptin pathway. *Molecular and Cellular Endocrinology* 200 99–109.

Persson E, Voznesensky OS, Huang YF & Lerner UH 2005 Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1 and C/EBP, but not NF-κB, in mouse calvarial osteoblasts. *Bone* 37 513–529.

Rotter V, Nagaev I & Smith U 2003 Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *Journal of Immunology* 162 4893–4902.

Verhasselt V, Buelens C, Willems F, De Groote D, Haeffner-Cavaillon N & Goldman M 1997 Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. *Journal of Immunology* 158 2919–2925.

Wiikberg JE & Mutulis F 2008 Targeting melanocortin receptors: an approach to treat weight disorders and sexual dysfunction. *Nature Reviews. Drug Discovery* 7 307–323.

Yoon SW, Goh SH, Chun JS, Cho EW, Lee MK, Kim KL, Kim JJ, Kim CJ & Poo H 2003 α-Melanocyte-stimulating hormone inhibits lipopolysaccharide-induced tumor necrosis factor-alpha production in leukocytes by modulating protein kinase A, p38 kinase, and nuclear factor κB signaling pathways. *Journal of Biological Chemistry* 278 32914–32920.

Zhao W, Liu M & Kirkwood KL 2008 p38alpha stabilizes interleukin-6 mRNA via multiple AU-rich elements. *Journal of Biological Chemistry* 283 1778–1785.

Received in final form 6 January 2010
Accepted 20 January 2010
Made available online as an Accepted Preprint 20 January 2010