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Melanocortin-4 receptor activation inhibits c-Jun N-terminal kinase activity and promotes insulin signaling

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Abstract

The melanocortin system is crucial to regulation of energy homeostasis. The melanocortin receptor type 4 (MC4R) modulates insulin signaling via effects on c-Jun N-terminal kinase (JNK). The melanocortin agonist NDP-MSH dose-dependently inhibited JNK activity in HEK293 cells stably expressing the human MC4R; effects were reversed by melanocortin receptor antagonist. NDP-MSH time- and dose-dependently inhibited IRS-1^{ser307} phosphorylation, effects also reversed by a specific melanocortin receptor antagonist. NDP-MSH augmented insulin-stimulated AKT phosphorylation in vitro. The melanocortin agonist melanotan II increased insulin-stimulated AKT phosphorylation in the rat hypothalamus in vivo. NDP-MSH increased insulin-stimulated glucose uptake in hypothalamic GT1-1 cells. The current study shows that the melanocortinergic system interacts with insulin signaling via novel effects on JNK activity.

Keywords

c-Jun N-terminal kinase (JNK); melanocortin receptor; AKT; insulin; insulin receptor substrate 1 (IRS-1); MT II; NDP-MSH; hypothalamus

1. Introduction

In the hypothalamus, melanocortin signaling is a key component of energy homeostasis. The melanocortinergic system consists of the prohormone proopiomelanocortin (POMC), its derivative melanocortin peptides (α -MSH, β -MSH, γ -MSH), adrenocorticotrophic hormone (ACTH), and the endogenous melanocortin peptide antagonists, agouti and agouti-related peptide (AgRP). In hypothalamic control of caloric intake, the relevant components of the melanocortin system include POMC-derived peptides, the antagonist AgRP, and melanocortin receptor types 3 and 4 (MC3R and MC4R) [9,12].

Melanocortin receptors belong to a superfamily of seven transmembrane G-protein coupled receptors. Conventional understanding of melanocortin receptor signaling involves the cyclic AMP transduction pathway via a G_s protein and adenylyl cyclase [1,13]. MC3R signaling has also been associated with changes in intracellular Ca^{2+} and inositol trisphosphate levels and with the protein kinase C pathway [20,26]. Previous studies have demonstrated that MC3R

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and MC4R activation can also stimulate extracellular signal-regulated kinases (ERK) activation, implying that multiple signal pathways may exist beyond the traditional cAMP pathway [6,7,10,33].

Growth factors, hormones, cytokines and environmental stressors such as ultraviolet radiation have the capacity to activate stress-activated protein/mitogen-activated kinase pathways (SAP/MAP). Protein kinases in these groups include extracellular signal-regulated kinases (ERK-1, -2 and -5), the four p38 isoforms (α , β , γ and δ) and the c-Jun-N-terminal kinase isoforms (JNK-1, -2 and -3). Numerous studies have demonstrated that SAP/MAP pathways regulate cellular proliferation, apoptosis and cellular differentiation, and members of these protein kinase families and upstream kinases have been implicated in a variety of human diseases [22,30,34]. Although initially identified by their ability to phosphorylate c-Jun in response to UV-irradiation, JNKs are now recognized to have a central role in obesity and obesity-related insulin resistance [15,31]. In the present study, interactions between the MC4R and insulin signaling pathways were demonstrated via alterations in JNK activity. Mediation of JNK activity by the melanocortinergic system provides a mechanism by which insulin signaling may be regulated within the hypothalamus.

2. Materials and Methods

2.1 Chemicals and Antibodies

NDP-MSH (α -MSH analogue, [Nle⁴, D-Phe⁷] α -MSH), MT II, and SHU9119 were purchased from Bachem (King of Prussia, PA). β -actin antibody and FLAG-M2 antibody-conjugated agarose were purchased from Sigma-Aldrich (St. Louis, MO). Phospho-AKT (Thr308) antibody and GST-c-Jun (1–89) were obtained from Cell Signaling Technologies (Beverly, MA). Rabbit HA antibodies were purchased from BD Clontech (Palo Alto, CA). Anti-phospho-IRS-1(Ser 307) was purchased from Upstate (Lake Placid, NY). HA-IRS-1 was provided by Dr. Liangyou Rui (Department of Molecular & Integrative Physiology, University of Michigan). FLAG-JNK1 vector was provided by Dr. Deepak Nihalani (Department of Internal Medicine, University of Michigan). Secondary IgG HRP antibody was purchased from Santa Cruz Biotechnology. Anisomycin and SP600125 were from Calbiochem (San Diego, CA).

2.2 Cell culture

HEK293 cells, stably transfected with the coding region of human MC4R gene expressed in pcDNA3.1 (Invitrogen, Carlsbad, CA), were used for this study. This cell line has previously been characterized [36,37]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin. GT1-1 cells (a generous gift from Dr. Richard I. Weiner, University of California-San Francisco) were cultured in DMEM with 10% FBS and 100 U/ml penicillin and 100 U/ml streptomycin. Cells were plated on 100 mm dishes and maintained at 37°C in a water-saturated atmosphere of 95% O₂ and 5% CO₂.

2.3 JNK activity assay

The JNK immunocomplex kinase assay was performed as previously described [5,24]. Plasmid (1 μ g/well) encoding Flag-JNK1 was transfected into HEK293 cells expressing MC4R in six well plates. Forty hours after transfection, cells were exposed to serum-free medium overnight. After treatment, cells were lysed in PK buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1mM Na₃VO₄, 50 mM NaF, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Sigma)]. Equal amounts of lysate proteins from each well were incubated with M2 monoclonal antibody conjugated to agarose for 18 hr at 4°C. Beads were washed twice with PK lysis buffer and twice with kinase buffer (25 mM HEPES, pH 7.4, 20 mM MgCl₂, 0.5 mM EGTA, 12.5 mM β -glycerophosphate, 0.1 mM orthovanadate, 0.5 mM

NaF). The complex was incubated for 30 min at 30°C in 30 µl of kinase buffer containing 20 µM ATP, 5 µCi [γ - 32 P]ATP, and 2 µg GST-c-Jun (1–89). The reaction was terminated by adding 10 µl 4 × SDS loading buffer and heating at 80°C for 5 min. The reaction mixture was subjected to SDS-PAGE electrophoresis, transferred to polyvinylidene membrane and exposed to X-ray film.

2.4 Assay of phosphorylation of IRS-1 at Serine 307

HEK293 cells expressing MC4R were cultured in six well plates. Cells were transfected with plasmid (1 µg/well) encoding Flag-JNK1 and HA-IRS-1 (1 µg/well). Forty hours after transfection, cells were exposed to serum-free medium for 16 hours. After treatment, cell lysates were used to assay for phospho-IRS-1^{ser307} by Western blotting.

Western blotting—Protein samples from cell lysates (15–20 µg) were subjected to electrophoretic separation on a 10% polyacrylamide gel (BioRad) and then transferred onto ImmobilonTM-P PVDF membrane (Millipore Corp., Bedford, MA). Blots were blocked at room temperature for 1 h in 5% milk in TBS-Tween 20 (0.05%) and then incubated overnight in primary antibody diluted 1/1000. Membranes were washed three times in TBS-Tween (0.05%) and then incubated for 1 h with secondary antibody, diluted 1/5000 in 5% TBS-Tween (0.05%). Detection was performed using Lumi-Light Blotting Substrate (Roche, Indianapolis, IN).

2.5 Assay of phosphorylation of AKT

AKT, a downstream signaling molecule of PI3K, was detected by Western blot analysis using phospho-AKT antibody.

2.6 Glucose uptake

The hypothalamic neuronal cell line GT1-1 was maintained in DMEM supplemented with 10% fetal bovine serum. Cells were cultured for 24 hours, then media was changed to serum-free DMEM containing 5 mM glucose and 1% bovine serum albumin for 2 hr at 37°C.

Before glucose transport measurements, cells were washed with KRH buffer (20 mM HEPES (pH 7.4), 1.25 mM MgSO₄, 1.25 mM CaCl₂, 136 mM NaCl, 4.7 mM KCl, and 0.1% bovine serum albumin). Glucose transport was determined using KRH buffer containing 0.1 mM 2-deoxyglucose and 0.5 µCi of 2-[1,2- 3 H]-deoxy-D-glucose (Sigma, St. Louis, MO). Nonspecific uptake, measured in the presence of 10 µM cytochalasin B, was subtracted from measured values. Glucose transport experiments were terminated after 10 min by aspiration and three washes with ice-cold phosphate-buffered saline. Cells were lysed in 0.1% SDS in phosphate-buffered saline and sonicated. Radioactivity was determined by scintillation counting [23,24].

2.7 ICV cannulation

Sprague Dawley rats with a body weight of 280–300g were used. Rats were anaesthetized with ketamine/xylazine and placed on a stereotaxic device with the incisor bar 3.3 mm below the interaural line according to Paxinos and Watson [28]. A stainless steel 26 gauge guide cannula was implanted into the third ventricle using the following stereotaxic coordinates: 2.2 mm posterior to the bregma, 8.2 mm ventral to the surface of the skull and directly along the midline. The cannula was anchored to the skull with screws and dental cement. An internal cannula (17 mm) was placed into the guide cannula to maintain patency. Rats were allowed to recover for 1 week. Guide cannula patency was assessed by injection of 10 ng angiotensin II in 5 µl of saline. Cannulas were considered patent if rats consumed at least 5 ml of water within 1 hr of injection. Rats with correct third ventricle cannulation were used five days later.

2.8 Insulin and MT II injection

The rats were injected with either MT II (50 ng/rat), insulin (1.3 mU), both MT II and insulin (in 5 μ l of saline), or saline into the third ventricle through a guide cannula [8,27]. The length of the injection needle is 17 mm. The rats were sacrificed after 20 minutes. Hypothalamus was rapidly dissected, placed into a 1.5-ml microcentrifuge tube with 250 μ l ice-cold lysis buffer, and homogenized using a Dounce homogenizer. Homogenates were centrifuged and supernatants were used to measure phospho-AKT by Western blot analysis.

2.9 Data analysis

Experiments were performed at least three times. For Western blots, analysis of densitometry was performed using Kodak 1D 3.6 software (Eastman Kodak, New Haven, CT). Data was analyzed using Graphpad Prism 4.0 (Graphpad Software, San Diego, CA), and expressed as mean $\pm$ SEM. Differences were analyzed by unpaired two-tailed Student's *t* test. A value of $p < 0.05$ was taken as significant.

3. Results

3.1 Effect of MC4R activation on JNK activity

HEK293 cells expressing MC4R were transfected with a Flag-JNK vector. The melanocortin agonist NDP-MSH (0.1 μ M) inhibited both basal and insulin-stimulated (0.1 μ M) JNK activity (Figure 1). The inhibition of insulin-stimulated JNK activity by NDP-MSH was dose-dependent (Figure 2). The melanocortin receptor antagonist, SHU9119, reversed the inhibitory effect of NDP-MSH on JNK activity (Figure 2). These results demonstrate that NDP-MSH-induced inhibition of JNK activity is a MC4R-mediated effect.

3.2 Effect of MC4R activation on IRS-1^{ser307} phosphorylation

It has been reported that JNK activation increases IRS-1^{ser307} phosphorylation [2]. HA-IRS-1 and Flag-JNK vector were transfected into HEK293 cells expressing MC4R. NDP-MSH inhibited IRS-1^{ser307} phosphorylation, reaching maximum at 20 and 30 minutes, and returning to control values at 60 minutes (Figure 3). NDP-MSH inhibition of IRS-1^{ser307} phosphorylation was dose-dependent over a range of 0.1 nM to 1 μ M (Figure 4). The melanocortin receptor antagonist SHU9119 dose-dependently reversed NDP-MSH-induced inhibition of IRS-1^{ser307} phosphorylation, indicating that the effect of NDP-MSH on IRS-1^{ser307} phosphorylation is mediated by MC4R (Figure 5).

3.3 Effect of JNK activation and inhibition on IRS-1^{ser307} phosphorylation

The above results are consistent with an interpretation that NDP-MSH inhibits JNK activity and IRS-1^{ser307} phosphorylation by activating MC4R. To examine the effects of JNK on IRS-1^{ser307} phosphorylation, the JNK activator, anisomycin, and the JNK inhibitor, SP600025, were utilized. NDP-MSH decreased anisomycin-stimulated IRS-1^{ser307} phosphorylation (Figure 6A). Inhibition of JNK by SP600125 decreased basal IRS-1^{ser307} phosphorylation in HEK293 cells expressing MC4R (Figure 6B), implying that direct JNK inhibition decreases IRS-1^{ser307} phosphorylation. The inhibitory effects of SP600025 were additive to those of NDP-MSH (Figure 6B).

3.4 Effect of NDP-MSH on insulin-stimulated AKT phosphorylation in vitro

MC4R activation inhibits IRS-1^{ser307} phosphorylation, implying that MC4R activation modulates insulin signaling. In order to further examine the effects of MC4R activation on insulin signaling, the effect of NDP-MSH on insulin-stimulated AKT phosphorylation was tested. NDP-MSH promoted insulin-stimulated AKT phosphorylation in HEK cells expressing

MC4R (Figure 7A). Similar results were observed in GT1-1 cells (Figure 7B). GT1-1 cells are derived from hypothalamus, and endogenously express MC4R [7, 19].

3.5 Effect of MT II on insulin-stimulated AKT phosphorylation in vivo

MT II and DNP-MSH are analogues of MSH. MT II has been widely used in vivo because of its high potency in vivo and ability to cross the blood-brain barrier [11]. They have the same downstream signaling mechanisms as the parent compound. Intracerebroventricular injection of insulin (1.5 mU) significantly increased AKT phosphorylation in rat hypothalamus. The melanocortin agonist, MT II (50ng), alone did not increase AKT phosphorylation, whereas administration of both insulin and MT II significantly enhanced AKT phosphorylation relative to insulin alone (Figure 8).

3.6 MC4R activation increases insulin-stimulated glucose uptake in GT1-1 cells

The effects of MC4R activation on insulin-stimulated glucose uptake were next investigated. Insulin (10^{-7} M) significantly increased glucose uptake in GT1-1 cells. NDP-MSH (10^{-7} M) alone did not affect basal glucose uptake, but the combination of NDP-MSH and insulin significantly increased glucose uptake relative to the effects of insulin alone (Figure 9).

4. Discussion

The melanocortinergic signaling system has important biological functions beyond feeding behavior. For example, MC3R and MC4R mediate inflammatory responses and regulate apoptosis [7,14,21]. The range of recognized biological actions is reflected in a diversity of signaling pathways. The current study demonstrates that melanocortin signaling regulates the activity of c-Jun NH₂-terminal kinases, and in turn, has effects on IRS-1 and AKT phosphorylation. NDP-MSH inhibited JNK activity in cells expressing human MC4R. NDP-MSH inhibited phosphorylation of IRS-1^{ser307}, effects that were dose-dependent, reversible by the antagonist SHU9119, and mimicked by direct JNK activation or inhibition. JNKs phosphorylate c-Jun at Ser⁶³ and Ser⁷³ as well as other transcription factors [18]. A variety of proliferative signals, cytokines and cellular stressors activate JNK-dependent pathways, but mediation of JNK activity by the melanocortinergic system has not been previously reported. Members of this protein kinase family have been implicated in human diseases, and this linkage has motivated the development of a growing number of small inhibitory molecules.

JNKs have been reported to have a role in obesity and obesity-related insulin resistance [15, 16]. In obesity-induced insulin resistance or type 2 diabetes, a defect in insulin signaling lies distal to the insulin receptor. Binding of insulin to the insulin receptor results in insulin receptor substrate (IRS-1) phosphorylation on tyrosine residues; tyrosine-phosphorylated IRS-1 recruits and activates various effectors that contain phospho-tyrosine binding domains [35]. In the context of obesity and systemic insulin resistance, IRS-1 may be phosphorylated at Ser³⁰⁷. Serine³⁰⁷ phosphorylation of IRS-1 interferes with its association with the insulin receptor, reduces tyrosine phosphorylation of IRS-1 in response to insulin and thereby suppresses downstream signaling and insulin action [2,3,17,29]. JNKs can phosphorylate IRS-1 on Ser³⁰⁷, and in this way, may contribute to the development of insulin resistance [2,3]. JNK also mediates feedback inhibition of insulin signaling. Insulin stimulates JNK activity; JNK promotes IRS-1 Ser³⁰⁷ phosphorylation and attenuates tyrosine phosphorylation. Inhibiting JNK enhances insulin signal transduction and glucose uptake. In this manner, insulin-activated JNK may act as a negative feedback regulator for insulin signaling [3,23]. JNK activity is increased in the hypothalamus of rats with Western diet-induced obesity [31]. In mouse models, suppression of JNK results in decreased adiposity [15].

During regulation of energy homeostasis, melanocortin receptors interact with other factors, such as leptin, cholecystokinin and neuropeptide Y [9,25,32]. Insulin is an important satiety factor, but direct regulation of insulin signaling by melanocortin peptides has not previously been reported. Banno et al reported that central administration of melanocortin agonist MT II improved insulin tolerance and increased the number of small-sized adipocytes in diet-induced obese rats, implying that melanocortin receptors may interact with insulin [4]. In the current study, MC4R activation increased insulin-stimulated AKT phosphorylation in vitro and in vivo and glucose uptake in vitro. NDP-MSH alone did not stimulate AKT phosphorylation, and did not significantly increase glucose uptake. The details of this interaction remain to be elucidated.

In conclusion, the present studies demonstrate that MC4R agonist enhances insulin signaling with a mechanism that involves inhibition of JNK activity, resulting in decrease of IRS-1^{ser307} phosphorylation and increase in AKT phosphorylation.

Acknowledgments

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Abbreviations

JNK	c-Jun N-terminal kinase
α-MSH	α -melanocyte stimulating hormone
NDP-MSH	[Nle ⁴ , DPhe ⁷]- α -melanocyte stimulating hormone
MAPK	mitogen-activated-protein kinase
MCR	melanocortin receptor
MT II	melanotan II/Ac-Nle-cyclo(-Asp-His-D-Phe-Arg-Trp-Lys-NH ₂)
SHU9119	Ac-Nle-c[Asp-His-D-Nal-Arg-Trp-Lys]-NH ₂
PI3K	Phosphatidylinositol 3-Kinase
IRS-1	insulin receptor substrate 1
IRS-1^{ser307}	insulin receptor substrate 1 at serine 307

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Fig. 1. NDP-MSH inhibits both basal and insulin-stimulated JNK activity

HEK 293 cells stably expressing human MC4R were transfected with Flag-JNK and grown for 48 hours, and then held overnight in serum-free media supplemented with 0.1 % BSA. After 10 minutes of treatment, cells were lysed and supernatants were subjected to JNK immunocomplex kinase assay. The optical density of each band was measured and normalized for protein loading. The blot is typical of an experiment that was replicated 3 times. * $p < 0.05$.

Insulin (0.1 μ M)	-	-	+	+	+	+
NDP-MSH (μ M)	-	-	-	0.01	0.1	0.1
SHU9119 (μ M)	-	0.1	-	-	-	0.1

pGST-c-Jun

 α Flag

Insulin (0.1 μ M)	-	-	+	+	+	+
NDP-MSH (μ M)	-	-	-	0.01	0.1	0.1
SHU9119 (μ M)	-	0.1	-	-	-	0.1

Fig. 2.

NDP-MSH inhibits insulin-stimulated JNK activity dose-dependently and this effect can be reversed by antagonist SHU9119. HEK 293 cells stably expressing human MC4R were transfected with Flag-JNK and cells were grown for 48 hours and then held overnight in serum-free media supplemented with 0.1 % BSA. After 10 minutes of treatment, cells were lysed and supernatants were subjected to JNK immunocomplex kinase assay. * $p < 0.05$ vs. control; + $p < 0.05$ vs. insulin alone; # $p < 0.05$ vs. insulin with 0.1 μ M NDP-MSH.

Fig. 3.

NDP-MSH inhibits IRS-1^{Ser307} phosphorylation. HEK 293 cells stably expressing human MC4R were transfected with Flag-JNK and HA-IRS-1. Cells were grown for 48 hours and then held overnight in serum-free media supplemented with 0.1 % BSA. After treatments, cells were lysed and supernatants were used to detect phospho-IRS-1^{Ser307} by Western blot analysis.

* $p < 0.05$ vs. Time = 0.

Fig. 4. NDP-MSH inhibits phosphorylation of IRS-1^{Ser307} in a dose-dependent manner. HEK 293 cells expressing MC4R were transfected with Flag-JNK and HA-IRS-1. Cells were incubated with NDP-MSH for 20 minutes. Phosphorylation of IRS-1^{Ser307} was measured by Western blot analysis.

Fig. 5.

MCR antagonist SHU9119 reverses NDP-MSH-induced inhibition of IRS-1^{Ser307}

phosphorylation. HEK 293 cells stably expressing human MC4R were transfected with Flag-JNK and HA-IRS-1. Cells were grown for 48 hours and then held overnight in serum-free media supplemented with 0.1 % BSA. After 20 minutes of treatment, cells were lysed and supernatants were used to detect phospho-IRS-1^{Ser307} by Western blot analysis. * $p < 0.05$ vs. control.

A **B** **Fig. 6.**

Effect of JNK activation and inhibition on IRS-1^{Ser307} phosphorylation. HEK 293 cells stably expressing human MC4R were transfected with HA-IRS-1. A. JNK activator anisomycin increased IRS-1^{Ser307} phosphorylation. * $p < 0.05$. Cells were preincubated with anisomycin for 30 minutes, then, with NDP-MSH for 20 minutes. B. JNK inhibitor SP600125 decreased IRS-1^{Ser307} phosphorylation. * $p < 0.05$ vs. control; # $p < 0.05$ vs. NDP-MSH. Cells were preincubated with SP600125 for 2 hours, then with NDP-MSH for 20 minutes.

Fig. 7.

NDP-MSH enhances insulin-stimulated AKT phosphorylation. HEK cells expressing MC4R and GT1-1 cells were cultured for 24 hours and then incubated with serum-free media supplemented with 0.1 % BSA overnight. Cells were incubated with insulin along with NDP-MSH for 20 minutes. Cells were lysed and supernatants were used to detect phosphorylation of AKT by Western blot analysis. NDP-MSH increases insulin-stimulated AKT phosphorylation both in HEK 293 cells (A) and GT1-1 cells (B). * $p < 0.01$ vs. Insulin 0.01 μ M.

Fig. 8.

MT II augments insulin-stimulated AKT phosphorylation in rat hypothalamus. Rats were injected with either MT II (50 ng/rat) (n=6), insulin (1.5 mU) (n=6), both MT II and insulin (n=6), or saline (n=6) into the third ventricle through a guide cannula. The rats were sacrificed after 20 minutes. Hypothalamus was rapidly dissected, and homogenized in cell lysis buffer. The supernatants were used to measure phospho-AKT by Westernblot analysis. * $p < 0.01$ vs. control; # $p < 0.05$ vs. Insulin alone.

Fig. 9. NDP-MSH increases insulin-stimulated glucose uptake in GT1-1 cells. Insulin (10^{-7} M) stimulates glucose uptake and NDP-MSH (10^{-7} M) increased insulin-stimulated glucose uptake. * $p < 0.05$ vs. control; # $p < 0.05$ vs. Insulin; $n=6$.