Molecular and Cellular Endocrinology ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Melanocortin 4 receptor activates ERK-cFos pathway to increase brain-derived neurotrophic factor expression in rat astrocytes and hypothalamus

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ARTICLE INFO

Article history:
Received 15 December 2014
Received in revised form 9 April 2015
Accepted 9 April 2015
Available online

Keywords:
MC4R
BDNF
ERK
RSK
cFos
PI3K
Src
Astrocytes
Hypothalamus

ABSTRACT

Melanocortins are neuropeptides with well recognized anti-inflammatory and anti-apoptotic effects in the brain. Of the five melanocortin receptors (MCR), MC4R is abundantly expressed in the brain and is the only MCR present in astrocytes. We have previously shown that MC4R activation by the α-melanocyte stimulating hormone (α-MSH) analog, NDP-MSH, increased brain-derived neurotrophic factor (BDNF) expression through the classic cAMP–Protein kinase A–cAMP responsive element binding protein pathway in rat astrocytes. Now, we examined the participation of the mitogen activated protein kinases pathway in MC4R signaling. Rat cultured astrocytes treated with NDP-MSH 1 μM for 1 h showed increased BDNF expression. Inhibition of extracellular signal-regulated kinase (ERK) and ribosomal p90 S6 kinase (RSK), an ERK substrate, but not of p38 or JNK, prevented the increase in BDNF expression induced by NDP-MSH. Activation of MC4R increased cFos expression, a target of both ERK and RSK. ERK activation by MC4R involves cAMP, phosphoinositide-3 kinase (PI3K) and the non receptor tyrosine kinase, Src. Both PI3K and Src inhibition abolished NDP-MSH-induced BDNF expression. Moreover, we found that intraperitoneal injection of α-MSH induces BDNF and MC4R expression and activates ERK and cFos in male rat hypothalamus. Our results show for the first time that MC4R-induced BDNF expression in astrocytes involves ERK-RSK-cFos pathway which is dependent on PI3K and Src, and that melanocortins induce BDNF expression and ERK-cFos activation in rat hypothalamus.

1. Introduction

Melanocortins are produced from pro-opiomelanocortin precursor cleavage. These neuropeptides include α-, β- and γ-melanocyte-stimulating hormones (MSH) and adrenocorticotropic hormone. Melanocortins are known to exert protective actions in the brain (Caruso et al., 2012b; Catania, 2008). There are five known melanocortin receptors (MCRs) designated MC1R through MC5R. Of the five known MCRs, **MC4R** is the only subtype expressed in rat astrocytes (Caruso et al., 2007; Selkirk et al., 2007). MC4R mediates antipyretic (Sinha et al., 2004), neuroprotective (Giuliani et al., 2006), and anorexigenic (Marsh et al., 1999) actions of α-MSH. MC4R activation reduces amnesia induced by interleukin-1β (Gonzalez et al., 2009) and it also improves cognitive performance in animals with impaired memory consolidation caused by interleukin-1β (Machado et al., 2010). We have previously shown that MC4R mediates the anti-inflammatory action of melanocortins in cultured astrocytes and in the hypothalamus (Caruso et al., 2004, 2007). More recently, we demonstrated that MC4R activation in astrocytes leads to increased brain-derived neurotrophic factor (BDNF) expression (Caruso et al., 2012a). BDNF is a neurotrophic factor essential for neuronal development, survival, and synaptic plasticity (Lewin and Barde, 1996) and has been proposed to participate in the anorexigenic effect of melanocortins in hypothalamus (Xu et al., 2003).

MC4R is a G-protein coupled receptor (GPCR) that through a classic signaling pathway activates adenylate cyclase (AC) leading to cAMP synthesis which in turn activates protein kinase A (PKA). Then PKA activates cAMP responsive element binding protein (CREB), which was shown to be activated by α-MSH in neurons (Caruso et al., 2010; Sutton et al., 2005). We recently showed that MC4R activation

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; AC, adenylate cyclase; BDNF, brain-derived neurotrophic factor; CREB, cAMP responsive element binding protein; DAPI, 4′,6-diamido-2-phenylindole dihydrochloride; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; HMB, hypothalamic fragment; HPRT, hypoxanthine–guanine phosphoribosyltransferase; MAPK, mitogen activated protein kinase; MCR, melanocortin receptor; NDP-MSH, [Nle(4),D-Phe(7)]melanocyte-stimulating hormone; PKA, protein kinase A; PI3K, phosphoinositide-3 kinase; PDK1, phosphoinositide-3-dependent protein kinase-1; Rp-cAMP, Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium; RSK, ribosomal p90 S6 kinase; RT-qPCR, reverse transcriptase- real time polymerase chain reaction.

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http://dx.doi.org/10.1016/j.mce.2015.04.008
0303-7207/

Please cite this article in press as: D. Ramírez, J. Saba, L. Carniglia, D. Durand, M. Lasaga, C. Caruso, Melanocortin 4 receptor activates ERK-cFos pathway to increase brain-derived neurotrophic factor expression in rat astrocytes and hypothalamus, Molecular and Cellular Endocrinology (2015), doi: 10.1016/j.mce.2015.04.008

in astrocytes increases BDNF expression in a cAMP-PKA-CREB dependent fashion (Caruso et al., 2012a). However, MC4R can also activate other signaling pathways that can contribute to its biological effects (Breit et al., 2011). Mitogen-activated protein kinases (MAPK) are a family of serine-threonine protein kinases involved in proliferation, migration and stress processes in the cell. MC4Rs activate the MAPK/extracellular signal-regulated kinase (ERK) when overexpressed in GT1-7 cells (Chai et al., 2006; Damm et al., 2012) and in vivo in the solitary nucleus (Sutton et al., 2005). Some reports also show that ERK activation may be either PKA-independent (Chai et al., 2006; Vongs et al., 2004) or dependent (Sutton et al., 2005). Also, it was shown that MC4R inhibits JNK activity when expressed in HEK293 cells (Chai et al., 2009) and inhibits p38 activation induced in a model of Alzheimer's disease (Giuliani et al., 2014). It was also suggested that MC4R may induce p38 activation in dorsal root ganglion (Chu et al., 2012). Therefore, MAPK involvement in MC4R action is dependent on the cell type where it is expressed. The phosphoinositide-3 kinase (PI3K)/Akt pathway is also involved in cell proliferation and survival. When activated, PI3K induces phosphoinositide-3-dependent protein kinase-1 (PDK1) activation by phosphorylation. Then, PDK1 phosphorylates other protein kinases such as Akt and p90 ribosomal protein S6 kinase (RSK) which can also be activated by ERK. Other studies also described that NDP-MSH, an analog of α-MSH, through MC4R or α-MSH through MC5R can induce ERK activation that is mediated by PI3K (Rodrigues et al., 2009; Vongs et al., 2004). However, MC4R signaling within the brain is still incompletely understood.

We previously showed that astrocytes express only MC4R and that its activation induces BDNF expression through cAMP-PKA-CREB pathway in these cells. In the present work we investigated MAPKs involvement in MC4R signaling in astrocytes. We found that ERK is the only MAPK activated by NDP-MSH through PI3K and Src. We showed that ERK-RSK-cFos pathway is activated by MC4R in astrocytes through which BDNF expression is increased. We also showed that ERK-cFos pathway is activated by melanocortins in vivo in rat hypothalamus as well.

2. Methods

2.1. Materials

α-MSH and [Nle(4),D-Phe(7)]melanocyte-stimulating hormone (NDP-MSH) were obtained from Bachem California (CA, USA). DMEM/F-12, antibiotics and glutamine were purchased from Invitrogen Life Technologies (CA, USA). Biotinylated donkey anti-mouse and anti-rabbit antibodies were obtained from Chemicon (Millipore, MA, USA). Phospho- ERK, phospho-Akt and total Akt antibodies were purchased from Cell Signaling Technology (MA, USA). Total ERK antibody was provided by Santa Cruz Biotechnology (CA, USA). All primers used were purchased from Invitrogen Life Technologies (CA, USA). Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium (Rp-cAMP), Forskolin, GSK233470, BRD7389, PP2 and 1,3-dihydro-1-(1-((4-(6-phenyl-1*H*-imidazo[4,5-g]quinoxalin-7-yl)phenyl) methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate (Akt1/2 inhibitor) were purchased from Sigma-Aldrich Corporation. SQ22536, LY294002, PD98059, SB203580, SP600125 were from Calbiochem. OSU-03012 was purchased from Selleck Chemicals. The concentrations of inhibitors used in this work are higher than the published IC50 concentrations except for OSU03012 and BRD7389, both used at the IC50 concentration. Protein kinase inhibitors can have detrimental effects on cell viability if they are used at high concentrations. Therefore, we tested whether the inhibitors used in this work affected astrocyte viability by MTT assay. On the basis of these results we selected the highest dose of the inhibitor that did not induce cell death of astrocytes (Table 1). All other media and supplements were obtained from Sigma-Aldrich Corporation, unless otherwise specified.

Table 1
Kinase inhibitors used in this study.

Target molecule	Inhibitor name	IC50	Dose used (μM)
MEK	PD98059	2–7 μM	10
p38	SB203580	50 nM	10
JNK	SP600125	40–90 nM	20
PI3K	LY294002	Up to 6.6 μM	20
Akt	Akt inhibitor	58–210 nM	0.5
AC	SQ22536	1.4–20 μM	100
PKA	Rp-cAMP	11 μM	20
PDK1	OSU03012	1–5 μM	1
PDK1	GSK2334470	10 nM	1
Src	PP2	0.73 μM	2
BRD	BRD7389	1 μM	1

2.2. Animals

Wistar rats were housed in a temperature-controlled facility at 25 °C on a 12 h light/12 h dark cycle with access to lab chow and water ad libitum. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the School of Medicine of the University of Buenos Aires, which are in line with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.3. Cell culture

Astrocytes were prepared from rat cerebral tissue of 1- to 2-day-old postnatal Wistar rat pups as described previously (Caruso et al., 2007). Cells were maintained in DMEM/F-12 medium containing 10% fetal bovine serum (PAA Laboratories GmBH, Pasching, Austria), 50 μg/ml streptomycin, 50 U penicillin in 75 cm^2 poly-L-lysine coated culture flasks at 37 °C in 5% CO_2. Cell culture medium was changed twice a week. Cells were trypsinized and subcultured and, after 2–3 days of stabilization, incubated with the drugs in MEM containing 2% fetal bovine serum, 6 mM L-glutamine, 50 μg/ml streptomycin, and 50 U penicillin. Cultures were routinely more than 95% pure astrocytes, as assessed by glial fibrillary acidic protein (GFAP) immunostaining.

2.4. In vivo experiments

Male Wistar rats 2 months old were randomly divided into two groups: (**3h**) rats were injected with α-MSH (0.5 mg/kg) or vehicle (saline) intraperitoneally (ip) once and killed 3 h later by decapitation; (**48h**) rats were injected with α-MSH (0.5 mg/kg) or vehicle ip twice a day for 2 days and killed by decapitation 48 h after the first injection. Then, a hypothalamic fragment containing the paraventricular, arcuate, dorsomedial and ventromedial nucleus (HMB) was dissected and immediately processed. In another set of experiments, animals treated for 48 h with α-MSH were anesthetized with ketamine (75 mg/kg) and xilazine (10 mg/kg) and transcardially perfused with 120 ml of PBS containing heparin followed by 120 ml of PBS–2% picric acid–4% paraformaldehyde. Brains were immediately removed and post-fixed in PBS–4% paraformaldehyde for 1 h. Then, the tissue was cryoprotected in 20% sacrose in PBS solution for 1–2 weeks. Coronal brain sections of 50 μm were cut with a cryostat (Leica Zeiss) following Paxino's rat brain atlas (Paxinos and Watson, 1997) for selection of the hypothalamus sections and the free floating sections were subjected to immunohistochemistry.

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