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α-Melanocyte-stimulating hormone modulates lipopolysaccharide plus interferon-γ-induced tumor necrosis factor-α expression but not tumor necrosis factor-α receptor expression in cultured hypothalamic neurons

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ABSTRACT

In a previous work we showed that the melanocortin alpha-melanocyte-stimulating hormone (α-MSH) exerts anti-inflammatory action through melanocortin 4 receptor (MC4R) *in vivo* in rat hypothalamus. In this work, we examined the effect of α-MSH on the expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and their receptors in primary cultured rat hypothalamic neurons. We also investigated α-MSH's possible mechanism/s of action. α-MSH (5 μM) decreased TNF-α expression induced by 24 h administration of a combination of bacterial lipopolysaccharide (LPS, 1 μg/ml) plus interferon-γ (IFN-γ, 50 ng/ml). Expression of TNF-α and IL-1β receptors TNFR1, TNFR2 and IL-1RI, was up-regulated by LPS + IFN-γ whereas α-MSH did not modify basal or LPS + IFN-γ-induced-TNFRs or IL-1RI expression. Both α-MSH and LPS + IFN-γ treatments increased CREB activation. α-MSH did not modify NF-κB activation induced by LPS + IFN-γ in hypothalamic neurons. In conclusion, our data show that α-MSH reduces TNF-α expression in hypothalamic neurons by a mechanism which could be mediated by CREB. The regulation of inflammatory processes in the hypothalamus by α-MSH might help to prevent neurodegeneration resulting from inflammation.

1. Introduction

Glial cells are believed to be the primary source of pro-inflammatory agents in the inflammatory response, while neuron participation in this process has been less extensively studied. Bacterial lipopolysaccharide (LPS) is a major inflammatory molecule that triggers the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in activated immune cells, and these cytokines play a critical role in mediating the inflammatory response of the host to infection and tissue injury (Hawiger, 2001). Although the production of pro-inflammatory cytokines has an important role in host defence against invading microbes, overexpression of cytokines may lead to inflammatory disorders (Han and Ulevitch, 2005). In the central nervous system (CNS), IL-1β and TNF-α are released from several cell types including astrocytes and microglia after brain injury. In addition, both IL-1β and TNF-α have direct influence on neurodegeneration (Thornton et al., 2006; Zhao et al., 2001), and inhibition of endogenous IL-1β and TNF-α protects against neuronal injury that occurs after cerebral ischemia (Meistrell et al., 1997; Relton and Rothwell, 1992). They are also highly expressed in Alzheimer's (Fillit et al., 1991; Griffin et al. 1998) and Parkinson's (Mogi et al., 1994, 1996) diseases.

TNF-α can activate two different receptors: TNFR1 and TNFR2. TNFR1 is constitutively expressed in dentate granule neurons and TNFR2 was also detected in these neurons (Harry et al., 2008). On the other hand, the biological effects of IL-1β are mediated by type I IL-1 receptor (IL-1RI) which has been detected in neurons after injury (Ericsson et al., 1995). Type II IL-1 receptor (IL-1RII) acts as a decoy receptor because it can bind IL-1β but does not lead to intracellular signalling (Sims et al., 1993).

The signalling pathways that regulate cytokine production have been studied intensively. The best described pathway involves a kinase cascade leading to activation of nuclear factor-κB (NF-κB) transcription factor (Li and Verma, 2002). Many signalling pathways have also been shown to activate the cyclic AMP responsive element binding protein (CREB) (Lonze and Ginty, 2002). This transcription factor is activated by cyclic AMP (cAMP)/protein kinase A (PKA)

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pathway by phosphorylation, activation that is sufficient to induce transcriptional activity of CREB. In addition, Ca++ signalling as well as ERK and p38 mitogen-activated protein kinase (MAPK) pathways can lead to CREB activation (Lonze and Ginty, 2002).

The neuropeptide α-melanocyte-stimulating hormone (α-MSH) is a 13 amino acid cleavage product of pro-opiomelanocortin hormone. The effects of α-MSH on immunity are well documented (Catania et al., 2004). Specifically, in the CNS, an immunoprivileged environment, it clearly has anti-cytokine effects and can modulate the production and action of pro-inflammatory cytokines (Catania et al., 2004; Lasaga et al., 2008). Centrally administered α-MSH is a potent antipyretic (Catania et al., 2004) and can also prevent damage in brainstem ischemia and reperfusion injury (Giuliani et al. 2006). A variety of effects of central IL-1β administration are blocked by α-MSH such as activation of the hypothalamic–pituitary–adrenal axis (Cragnolini et al., 2004; 2006a; 2006b) and the anxiogenic behaviour and memory impairment induced by this cytokine (Gonzalez et al., 2009).

Two of the five melanocortin receptor subtypes, melanocortin 3 receptor (MC3R) and MC4R, are extensively expressed in the brain and are thought to be the primary mediators of behavioural and immunomodulating effects of melanocortin peptides (Catania et al., 2004; Lasaga et al., 2008). We previously reported that α-MSH through MC4R reduced *in vivo* hypothalamic expression of inducible nitric oxide synthase and cyclooxygenase-2 induced by LPS (Caruso et al., 2004). We recently demonstrated that α-MSH decreases the release of nitric oxide and prostaglandins induced by LPS and interferon-γ (IFN-γ) in astrocytes through MC4R activation, reducing inflammatory response and also preventing apoptosis induced by LPS and IFN-γ in these cells (Caruso et al., 2007).

In the present study, we demonstrate the presence of MC3R and MC4R receptors in cultured hypothalamic neurons. We also determined that pro-inflammatory cytokine TNF-α and its receptors are up-regulated in hypothalamic neurons by treatment with LPS + IFN-γ whereas α-MSH reduces LPS + IFN-γ-induced TNF-α expression but failed to modify cytokine receptor expression. Although the underlying signalling mechanisms involved in this anti-inflammatory action are not clear, we suggest that α-MSH effects could be mediated by CREB activation.

2. Materials and methods

2.1. Reagents

LPS (*Escherichia coli*, serotype O127:B8) was purchased from Sigma-Aldrich Corporation (MO, USA). α-MSH was obtained from Bachem California Inc. (CA, USA). Interferon-γ (IFN-γ) was purchased from Boehringer Ingelheim, Argentina. Horse serum was obtained from PAA laboratories GmBH (Pasching, Austria). DMEM, antibiotics, N2 and B27 supplements and all RT-PCR reagents were purchased from Invitrogen Life Technologies (CA, USA) unless specified otherwise. Biotinylated donkey anti-mouse and anti-rabbit antibodies were obtained from Chemicon International Inc. (CA, USA). Anti-TNFR1, anti-TNFR2 and anti-IL-1R1 antibodies were purchased from Abcam (MI, USA). Anti-NF-κB p65 was obtained from BD Biosciences (CA, USA) and anti-IκBα was from Cell Signaling Technology (MA, USA). TNF-α, TNFR1, TNFR2, IL-1β, IL-1R1, and β-actin primers were purchased from Invitrogen Life Technologies (CA, USA). MC3R and MC4R primers were obtained from Integrated DNA Technologies Inc. (IA, USA). All other media and supplements were obtained from Sigma-Aldrich Corporation unless specified otherwise.

2.2. Cell culture

Pregnant Wistar rats were anesthetized and 16 day old fetuses were aseptically removed. Cerebral hemispheres were placed under a dissecting microscope and the ventromedial hypothalamic region (delimited by the optic chiasm, lateral hypothalamic sulcus and mammillary bodies) was dissected and stripped of meninges. At this early stage of development the ventromedial hypothalamic region contains the differentiating neurons for the ventromedial hypothalamic nucleus and the arcuate nucleus (Bayer and Altman, 1995). Tissue blocks were placed in 3 ml of 0.25% trypsin (Invitrogen Life Technologies) in phosphate-buffered saline (PBS) for 15 min at 37 °C. Cells were dissociated by repeated passage through a small-bored Pasteur pipette. The resulting cell suspension was centrifuged for 1 min at 1500 rpm and the cell pellet resuspended in 3 ml of DMEM-S (DMEM with Streptomycin 50 μg/ml, Penicillin 50 U/ml, 10% horse serum with N2 and B27 supplements). Cells were plated on poly-L-lysine coated culture dishes and maintained with DMEM plus 10% horse serum for 1 h. Then, the medium was replaced entirely by serum-free medium plus N2 mixture. Cell culture was kept in a humidified 37 °C incubator with 5% CO_2, then neurons were incubated with LPS 1 μg/ml + IFN-γ 50 ng/ml with or without α-MSH (5 μM) for 1 or 24 h. Purity of cultures was 91.68% ± 1.70 determined by immunofluorescence detection of neurons with MAP2 antibody in three different experiments with a total of 2500 cells (Fig. 1), as previously described by Ferreira and Cáceres (1991).

2.3. Immunocytochemistry

Neurons were identified by a polyclonal antiserum raised against anti-microtubule-associated protein 2 antibody (MAP2, 1:1000), and astroglial cells were identified by a monoclonal anti-glial fibrillary acidic protein antiserum (GFAP, 1:200). Cells were fixed for 30 min at room temperature with 4% (wt/vol) paraformaldehyde in PBS containing 4% (wt/vol) sucrose. Cells were washed with PBS, permeabilized with 0.2% (vol/vol) Triton X-100 in PBS for 5 min, and washed again with PBS. Cell were incubated with primary antibodies (1–3 h at room temperature), washed with PBS, and then incubated with Alexa 488 (dilution 1:600) or Alexa 564 (dilution 1:600) conjugated secondary antibodies (1 h at 37 °C), then washed with PBS and the coverslips mounted using FluorSave (Calbiochem, La Jolla, CA). Cells were visualized with an inverted Zeiss microscope, and images (8 bites) were collected using a CCD camera (Orca 1000, Hamamatsu Corp., Middlesex, NJ) and Metamorph software (Molecular Devices).

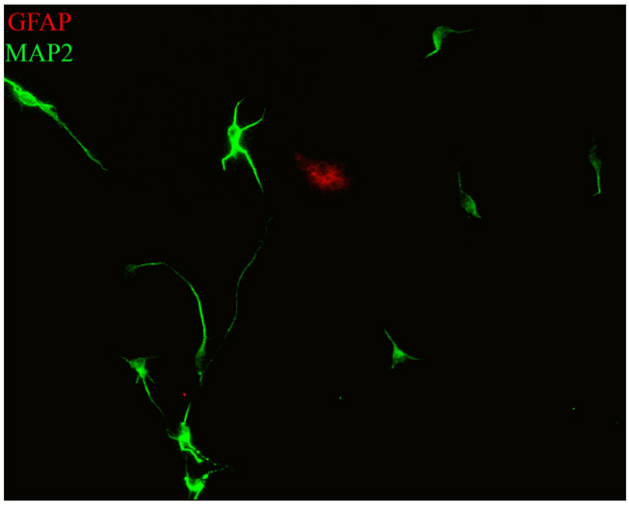

Fig. 1. Hypothalamic neurons in culture. Hypothalamic neurons were cultured in DMEM-S. Cells were identified using MAP2 (green) as neurons and using GFAP (red) antibody as glial cells.