Biochimica et Biophysica Acta 1812 (2011) 565–571

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Tumor necrosis factor-alpha regulates the Hypocretin system via mRNA degradation and ubiquitination

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ARTICLE INFO

Article history:
Received 14 October 2010
Received in revised form 10 November 2010
Accepted 12 November 2010
Available online 18 November 2010

Keywords:
Hypocretin
Orexin
Tumor necrosis factor
Sleep disorder
Hypocretin receptor
Orexin receptor

ABSTRACT

Recent studies recognize that Hypocretin system (also known as Orexin) plays a critical role in sleep/wake disorders and feeding behaviors. However, little is known about the regulation of the Hypocretin system. It is also known that tumor necrosis factor alpha (TNF-α) is involved in the regulation of sleep/wake cycle. Here, we test our hypothesis that the Hypocretin system is regulated by TNF-α. Prepro-Hypocretin and Hypocretin receptor 2 (HcrtR2) can be detected at a very low level in rat B35 neuroblastoma cells. In response to TNF-α, Prepro-Hypocretin mRNA and protein levels are down-regulated, and also HcrtR2 protein level is down-regulated in B35 cells. To investigate the mechanism, exogenous rat Prepro-Hypocretin and rat HcrtR2 were overexpressed in B35 cells. In response to TNF-α, protein and mRNA of Prepro-Hypocretin are significantly decreased (by 93% and 94%, respectively), and the half-life of Prepro-Hypocretin mRNA is decreased in a time- and dose-dependent manner. The level of HcrtR2 mRNA level is not affected by TNF-α treatment; however, HcrtR2 protein level is significantly decreased (by 86%) through ubiquitination in B35 cells treated with TNF-α. Downregulation of cellular inhibitor of apoptosis protein-1 and -2 (cIAP-1 and -2) abrogates the HcrtR2 ubiquitination induced by TNF-α. The control green fluorescent protein (GFP) expression is not affected by TNF-α treatment. These studies demonstrate that TNF-α can impair the function of the Hypocretin system by reducing the levels of both Prepro-Hypocretin and HcrtR2.

1. Introduction

The neuropeptides, Hypocretin-1 and Hypocretin-2 (also known as Orexin-A and Orexin-B), were first described in 1998 by two research groups [1,2]. Hypocretin-1 and Hypocretin-2 are derived from a single precursor, the Prepro-Hypocretin, through proteolytic cleavage [1,2]. Prepro-Hypocretin is expressed mainly in hypothalamic neurons, as well as in the testis [1–3]. Two Hypocretin receptors, the Hypocretin receptors 1 and 2 (HcrtR1 and HcrtR2), have been identified so far, and they share 64% of homology [2,4,5]. HcrtR1 and HcrtR2 are expressed in multiple organs, such as the brain, kidney, lung, and testis [3,4]. Prepro-Hypocretin and Hypocretin receptors are highly conserved among species [1,4,6,7]. Growing evidence supports that homeostasis of the Hypocretin system is important for the integrity of sleep/wakefulness cycle, feeding behavior, and emotion [8–11]. Dysregulation of the Hypocretin system has been blamed for the sleep disorder, narcolepsy, as well as emotion disorders, such as depression [4,9,11,12].

Tumor necrosis factor-alpha (TNF-α) is well known as a pro-inflammation cytokine, and plays an important role in host defense and pathogenesis of various diseases [13–15]. It is a 185 amino acid glycoprotein cytokine, and was identified in 1975 [16]. TNF-α signaling cascade is propagated by binding to TNF receptors, and, upon activation, TNF receptors bind to TNF receptor-associated factors and other downstream signaling proteins, resulting in regulation of gene expression and cell functions [13–15]. TNF-α has been associated with the development of multiple brain disorders, such as depression, narcolepsy, multiple sclerosis, Alzheimer's disease, and Parkinson's disease [17–20]. TNF-α is also called as adipokine due to its role in obesity and diabetes [21]. Published data indicate that TNF-α is involved in the regulation of sleep/wakefulness cycle and fatigue during infections [22]; but, the mechanism is not completely understood.

In the present study, we demonstrate that TNF-α can impair the Hypocretin system through a decrease of mRNA half-life and protein

Abbreviations: TNF-α, tumor necrosis factor alpha; HcrtR2, Hypocretin receptor 2; cIAP, cellular inhibitor of apoptosis protein; FAK, focal adhesion kinase; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcriptase polymerase chain reaction; MSCV vector, murine stem cell viral vector; GFP, green-fluorescent-protein; mRNA, messenger RNA

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doi:10.1016/j.bbadis.2010.11.003

ubiquitination. TNF-α treatment decreases the half-life of the Prepro-Hypocretin mRNA, resulting in downregulation of both protein and mRNA levels of Prepro-Hypocretin. TNF-α treatment decreases HcrtR2 protein level through a ubiquitination pathway mediated by cellular inhibitor of apoptosis protein-1 and -2 (cIAP-1 and cIAP-2), without affecting the mRNA level of HcrtR2. These data for the first time provide evidence that TNF-α can directly downregulate the Hypocretin system, suggesting a role of TNF-α in the development of disorders caused by the impaired Hypocretin system.

2. Materials and methods

2.1. Reagents

Recombinant tumor necrosis factor alpha (TNF-α) was obtained from R&D Systems (Minneapolis, MN). The following purified polyclonal antibodies were purchased: anti-Prepro-Hypocretin (Milliopore, Billerica, MA), anti-HcrtR2, anti-focal adhesion kinase (FAK), and anti-Green Fluorescent Protein (GFP) (Santa Cruz Biotechnology, Santa Cruz, CA). The following purified monoclonal antibodies (mAb) were purchased: anti-glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Research Diagnostics, Flanders, NJ) and anti-human/mouse pan cellular inhibitor of apoptosis protein-1 and -2 (cIAP1 and cIAP2)-specific antibody which was obtained from R&D Systems (Minneapolis, MN). All other reagents were purchased from Thermo Fisher Scientific (P-SUWANEE, GA), Sigma-Aldrich (St. Louis, MO), or Bio-Rad (Hercules, CA).

2.2. Cell culture and MSCV retroviral vectors

The B35 rat neuroblastoma cells were derived from the central nervous system, and obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained and propagated in Dulbecco's modified Eagle's medium(DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin/gentamycin as described [23]. Stable cell lines expressing Prepro-Hypocretin and HcrtR2 were established using the MSCV Retroviral Expression Vector System (with the Neomycin resistance gene) purchased from Clontech (Mountain View, CA) and used according to the manufacturer's instructions. Briefly, rat Prepro-Hypocretin cDNA (gene accession #AF041241) or rat HcrtR2 cDNA (gene accession #AF041246) was first inserted into the MSCV vectors between the EcoRI site and the XhoI site of the polylinker located within the multiple cloning sites of the MSCV vector. Then, the MSCV-Prepro-Hypocretin vector or MSCV-HcrtR2 vector was transfected into the packaging cells (Clontech) by liposome-mediated transfections (Lipofectamine 2000, from Invitrogen, Carlsbad, California) to produce infectious, but replication-incompetent, retroviral particles, and the collected supernatants containing retroviral particles were used to transfect the B35 cells. Stable clones were selected by Neomycin as the MSCV vector contains the Neomycin resistance gene. The green-fluorescent-protein (GFP) protein was also subcloned into MSCV vector, and MSCV-GFP was used as a control and also used to optimize the transfection and selection conditions. GFP-positive cells were easily detected under a fluorescent microscope (Nikon, Japan, Model: Eclipse, Ti).

2.3. Western blotting and immunoprecipitation

This was essentially as previously described by us [23]. Briefly, cells were lysed in 1% NP-40 lysis buffer containing the following inhibitors, 100 μM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM sodium vanadate, and 20 μg/ml TLCK. Protein concentration of whole cell or whole lung lysate was determined by BCA kit (Pierce, Rockford, IL). Equivalent micrograms of whole cell lysates was electrophoresed on SDS PAGE, transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), probed or stripped followed by re-probing with indicated antibodies, and developed with ECL system (Pharmacia Biotech, Piscataway, NJ). Ubiquitinated HcrtR2 level was determined by Western blotting under non-disulfide-reduced conditions following immunoprecipitation of equivalent cell lysates as described previously [24]. The expression of G3PDH protein was used as a loading control. Quantitative analysis (densitometry) of Western blots was performed by calculating the relative density (pixel density) of the immunoreactive bands after acquisition of the blot image (scanning) and analysis with Adobe Photoshop software as described [23]. The background of densitometric reading on the ECL-developed film was subtracted.

2.4. siRNA studies

Transfection of siRNA was done as described previously [24]. Commercially available small interfering RNA (siRNA) duplexes directed against rat cIAP1 and cIAP2 were purchased from Invitrogen (Carlsbad, California) as described [25], and are all selected and validated Stealth siRNAs. Control siRNA duplexes were purchased from Dharmacon (Lafayette, CO). Lamin A/C siRNA (Dharmacon) was used to optimize the transfection conditions and efficiency. Additional controls of nonspecific siRNA duplex or vehicle alone were evaluated and found not to alter cIAP-1 and cIAP-2 mRNA and protein expression at the concentration of siRNA found to reduce the specific target message and protein optimally.

2.5. RT-PCR and real-time quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), and 1 μg of total RNA was reverse transcribed to cDNA for RT-PCR and real-time quantitative RT-PCR as described previously [26]. Quantitative RT-PCR analysis was carried out with the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) using the Biorad iQ5 Real-Time PCR Detection System, according to the manufacturer's instructions. The quantitative RT-PCR was performed using 4 l of the synthesized cDNA, 12.5 μl of SYBR® Green PCR Master Mix, 1 μl of primer, and 7.5 μl of water in a final 25 μl volume. Samples were assayed in triplicate, and the values were normalized to the relative amounts of beta-actin mRNA level. Primer sequences were as described [3]. Primers for Prepro-Hypocretin: sense 5′-GCCGTCTCTACGAACTGTTG-3′ and antisense 5′-GAGGAGAGGGGAAAGTTAG-3′; for HcrtR2: sense 5′-CAATGTTGTTGGGGTGCTTA-3′ and antisense 5′-TCCCCCTCTCA-TAAACTTGG-3′; for rat focal adhesion kinase (FAK) (gene accession #NM_013081): sense 5′-CCTTAACAATGCGCCAGTTT-3′ and antisense 5′-CCAGATACGCGAGTGCTGTA-3′; and for Beta-actin: sense 5′-GTGGGTATGGGTCAGAAGGA-3′ and antisense 5′-AGCGCGTAACCCTCA-TAGAT-3′. All primers were purchased from Fisher Scientific.

2.6. mRNA stability assay

mRNA half-life was determined by culturing B35 cells in a medium with actinomycin D (10 μg/ml) to block transcription as described by Lindholm et al. [27]. Briefly, immediately after addition of actinomycin D, B35 cells were treated with TNF-α or vehicle for the indicated time, and harvested. Total RNA was collected, and the amounts of Prepro-Hypocretin or HcrtR2 mRNA at each time point were quantified by real-time RT-PCR, and normalized to the amounts of β-actin mRNA at the same time point as described [26].

2.7. Statistical analysis

Data were analyzed using the unpaired or paired t-test analysis (for comparisons between two groups) (Sigma Plot, SPSS Inc.), and expressed as means ± SE. Experiments were performed two to four times with duplicates. Linear regression was performed by using