Biochimica et Biophysica Acta 1833 (2013) 1476–1488

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Inhibition of c-Jun NH_2-terminal kinase stimulates mu opioid receptor expression via p38 MAPK-mediated nuclear NF-κB activation in neuronal and non-neuronal cells

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ARTICLE INFO

Article history:
Received 1 October 2012
Received in revised form 2 February 2013
Accepted 18 February 2013
Available online 26 February 2013

Keywords:
mu opioid receptor
JNK
MAPK
NF-κB

ABSTRACT

Despite its potential side effects of addiction, tolerance and withdrawal symptoms, morphine is widely used for reducing moderate and severe pain. Previous studies have shown that the analgesic effect of morphine depends on mu opioid receptor (MOR) expression levels, but the regulatory mechanism of MOR is not yet fully understood. Several *in vivo* and *in vitro* studies have shown that the c-Jun NH_2-terminal kinase (JNK) pathway is closely associated with neuropathic hyperalgesia, which closely resembles the neuroplastic changes observed with morphine antinociceptive tolerance. In this study, we show that inhibition of JNK by SP600125, its inhibitory peptide, or JNK-1 siRNA induced MOR at both mRNA and protein levels in neuronal cells. This increase in MOR expression was reversed by inhibition of the p38 mitogen-activated protein kinase (MAPK) pathway, but not by inhibition of the mitogen-activated protein/extracellular signal-regulated kinase (MEK) pathway. Further experiments using cell signaling inhibitors showed that MOR upregulation by JNK inhibition involved nuclear factor-kappa B (NF-κB). The p38 MAPK dependent phosphorylation of p65 NF-κB subunit in the nucleus was increased by SP600125 treatment. We also observed by chromatin immunoprecipitation (ChIP) analysis that JNK inhibition led to increased bindings of CBP and histone-3 dimethyl K4, and decreased bindings of HDAC-2, MeCP2, and histone-3 trimethyl K9 to the MOR promoter indicating a transcriptional regulation of MOR by JNK inhibition. All these results suggest a regulatory role of the p38 MAPK and NF-κB pathways in MOR gene expression and aid to our better understanding of the MOR gene regulation.

1. Introduction

Opiate drugs exert their effects through three major types of opioid receptors: mu, delta and kappa [1]. These receptors in the brain are activated by endogenous peptides such as enkephalins, dynorphins and endorphins, which are released by neurons. Opioid receptors can also be activated by exogenous alkaloid opiates, the prototype of which is morphine, which remains the most valuable painkiller in contemporary medicine. Although the three opioid receptor genes are highly homologous in their coding exons, their amino-terminal and caboxy-terminal ends are diverse, and these regions govern the unique ligand-binding and signal transduction properties of each receptor [2]. Experiments with transgenic and knockout mice have clearly demonstrated the role of mu opioid receptor (MOR) in morphine's pharmacological effects, including analgesia, physical dependence, and tolerance [3–5].

The mouse gene that encodes MOR (*oprm1*) is located on chromosome 10 and covers a length of 250 kilobases (kb). MOR transcription can start from the distal promoter (DP) or the proximal promoter (PP) [6]. PP transcripts are preferentially used in most tissues and cultured cells and account for most MOR activity. The DP and PP are both TATA-less and GC-rich, and they contain binding sites for multiple

Abbreviations: MOR, mu opioid receptor; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; NF-κB, nuclear factor-kappaB; DP, distal promoter; PP, proximal promoter; Oct-1, octamer-1; SOX, sry-related high-mobility-group box; PCBP, poly(C) binding protein; SP1, specificity protein 1; AP2, activator protein 2; CREB, cAMP response element binding protein; SAPK, stress-activated protein kinase; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; DRG, dorsal root ganglion; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one; QNZ, 6-amino-4-(4-henoxyphenylethylamino)quinazoline; SB203580, 4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; LY294002, 2,(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-amino phenylthio]butadiene; PDTC, pyrrolidine dithiocarbonate; PI3-K, phosphoinositide 3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; MeCP2, methyl-CpG-binding protein 2; H3dmK4, histone 3-dimethyl lysine 4; H3me3K9, histone 3-trimethyl lysine 9; HDAC, histone deacetylase; aceH, acetyl-histone; Brg1, Brm-related gene 1; NRSF, neuron-restrictive silencing factor; Dnmt1, DNA methyltransferase

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http://dx.doi.org/10.1016/j.bbamcr.2013.02.017

regulatory elements such as Oct-1 [7], IL-4 response element [8], SOX [9,10], PU.1 [11,12], PCBP [13], SP1 [14], AP2 [14–17], NF-κB [18], and CREB [19]. In addition to the DP and PP, the mouse gene also uses a TATA-containing promoter known as E11, located more than 10 kb upstream of the translation start site [1]. Several studies have shown that transcription of the MOR gene can be regulated by fentanyl, morphine, interleukin-1, lipopolysaccharide, protein synthesis inhibitors (cycloheximide, anisomycin and puromycin), dopaminergic drugs, histone deacetylase inhibitors, and demethylating agents [19,20]. However, the molecular events that lead to changes in MOR gene expression have just begun to be explored.

JNK (c-Jun NH_2-terminal kinase), a serine threonine protein kinase, is a member of the mitogen-activated protein kinase (MAPK) family and includes three genes, *jnk1, jnk2,* and *jnk3*. JNKs are a type of stress-activated protein kinase (SAPK), and can be activated by various cellular stresses such as heat shock, DNA damage, a rise in intracellular reactive oxygen species and calcium influx, neurodegeneration, and proinflammatory cytokines (such as tumor necrosis factor-alpha[TNF-α], interleukin-6 [IL-6], interleukin-1beta [IL-1β], interferon-gamma [IFN-γ]) [21]. JNKs have been implicated in processes such as oncogenic transformation, apoptosis, and neurodegeneration [22]. Of the three JNK members, JNK-3 is predominantly found in the brain and has different functions than JNK-1 and JNK-2. SP600125 (SP) is an anthrapyrazole and a reversible ATP-competitive inhibitor of JNK-1, JNK-2 and JNK-3; it has been successfully used *in vivo* and *in vitro* to block JNK activation [23].

Chronic morphine treatment has been shown to activate JNK in SH-SY5Y cells [24,25], T cells [26], and spinal cord [27]. In a rat model, single or chronic morphine injections induce JNK-3 mRNA in the frontal cortex and after cessation of morphine treatment, sustained elevation of JNK-3 mRNA expression occurs in the hippocampus and thalamus [28]. Moreover, MOR desensitization and acute analgesic tolerance to morphine and related opiates were blocked by JNK inhibition [27,29]. In L5-spinal nerve ligation pain models, transient JNK activation increases in dorsal root ganglion (DRG) neurons followed by a persistent activation in spinal astrocytes which contributes to the maintenance of neuropathic pain symptoms [21,30]. In these animal pain models, selective inhibition of JNK inhibits mechanical allodynia and heat hyperalgesia [30,31]. Collectively, these results suggest a role for JNK in the pharmacological effects of nociception and opioid systems. In our previous efforts to identify the signaling events in transcriptional activation of the MOR gene, we observed that SP treatment of P19 cells significantly increases MOR mRNA expression [20]. In this study, we investigate the molecular mechanism that leads to expression of the MOR gene upon JNK inhibition.

2. Materials and methods

2.1. Materials

SP600125 (SP), cell-permeable JNK inhibitor, and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ) were purchased from EMD Biosciences (San Diego, CA). 2,(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002 (LY)), wortmannin and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) were purchased from Cell Signaling Technology (Beverly, MA). 4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580 (SB)), actinomycin-D (act-D), and pyrrolidine dithiocarbonate (PDTC) were purchased from Sigma (St Louis, MO). Anti-MOR antiserum was generated in rabbits by injecting GST-fused MOR protein containing amino acids 340–398 of the MOR C-terminus. The specificity of the antiserum was confirmed in flow cytometry analysis of HEK 293 T cells and P19 cells stably expressing MOR. Anti-phospho-c-Jun, anti-phospho-SAPK/JNK, anti-JNK-1, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-AKT, anti-AKT, anti-phospho p42/p44 MAPK, anti-p42/44 MAPK, anti-phospho-p65 (Ser 536), anti-phospho CREB, anti-phospho MSK1 (Thr 581) antibodies were obtained from Cell Signaling Technology (Beverly, CA). Anti-c-Jun, anti-c-fos, anti-p65, anti-phospho-p65 (Ser 276), and anti-p50 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho serine antibodies and anti-CREB were obtained from Millipore (Billerca, MA). Anti-histone-dimethyl lysine 4 and anti-histone-trimethyl lysine 9 antibodies were obtained from Abcam (Cambridge, MA). Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse IgG were supplied by BioRad (Hercules, CA). Alexa Fluor 488-conjugated goat anti-rabbit were purchased from Invitrogen (Carlsbad, CA). Other reagents for molecular studies were supplied by Sigma Chemicals (St. Louis, MO).

2.2. Cell culture and transfection

P19 cells were cultured and differentiated as described previously [32]. For treatments, 5×10^5 cells were seeded into each wells of a 6-well plate one day before treatment. Cells were treated for 6 h with SP (25 μM), SB (25 μM), LY (25 μM), U0126 (10 μM) and QNZ (10 nM), and total RNA was harvested for RT-PCR. For transfection, 1×10^5 cells were seeded in 12-well dishes and co-transfected the next day for 24 h with the MOR promoter construct and a one-fifth molar ratio of pCH110 (for β-galactosidase assay) using effectene transfection reagent (Qiagen, Valencia, CA) as described previously [10]. Cells were treated with SP for 12 h, and cell lysates were analyzed for firefly luciferase activity and β-galactosidase activity as described by the manufacturer's protocol (Promega and Tropix, respectively). Results were expressed as relative luciferase activity compared to the control cells. For siRNA transfection, cells were seeded as above and transfected on the following day with 50 nM of control siRNA, JNK-1 siRNA, JNK-2 siRNA (Santa Cruz, CA) or SignalSilence NF-κB p65 siRNA (Cell Signaling Technology) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) as suggested by the manufacturer's protocol. After 36 h of transfection, cells were treated as required and harvested for western blotting and RT-PCR. NMB neuroblastoma cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), and HEK 293 T cells were cultured in advanced DMEM medium containing 5% FBS supplemented with Glutamax (Gibco, Invitrogen). Cells were maintained at 37 °C in a humidified incubator and sub-cultured every 2–3 days as required.

2.3. RT-PCR and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) and analyzed by RT-PCR using the MOR gene-specific primers mMOR-S and mMOR-AS [32]. Semi-quantitative RT-PCR was performed in 200 ng–1 μg of total RNA using a Qiagen OneStep RT-PCR kit (Valencia, CA). Similar reactions were performed using β-actin as an internal control [32]. qRT-PCR was performed as described previously [33] using the same MOR primer set and the Quantitect SYBR Green RT-PCR kit (Valencia, CA). Relative mRNA expression was analyzed as described previously [32]. The number of target molecules was normalized against that obtained for β-actin, used as an internal control. The specificity of qRT-PCR primers was determined using a melt curve after the amplification to show that only a single species of PCR product resulted from the reaction. The PCR products were also verified on an agarose gel. The RT-PCR and qRT-PCR experiments were repeated at least three times to obtain statistical significance.

2.4. Western blotting and immunoprecipitation

Western blotting was performed as previously described [20]. 4×10^5 cells were seeded into each well of 6-well dishes and treated as required. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in buffer composed of 50 mM Tris–Cl,