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Role of CDK5 in neuroprotection from serum deprivation by μ-opioid receptor agonist

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Abstract

Cyclin-dependent kinase 5 (CDK5), a unique member of the CDK family of cyclin-dependent kinases, is predominantly expressed in postmitotic neurons with proposed roles in both cell survival and programmed cell death. To understand how CDK5 participates in such disparate cellular outcomes, we investigated whether activation of CDK5 could mediate neuroprotection from serum deprivation by μ-opioid receptor agonist in differentiated SH-SY5Y cells and primary hippocampal neurons. We found that CDK5 kinase activity decreased following serum deprivation in differentiated SH-SY5Y cells coincident with increased cell loss and activation of caspases cascade activation, which was reversed by opioid antagonist. Overexpression of CDK5 in serum-free medium reversed activation of caspase cascade and augmented DAMGO neuroprotection. Blocking CDK5 activity by pharmacologic inhibitor, roscovitine or overexpression of dominant negative CDK5 augmented activation of cell death markers and diminished μ-opioid receptor agonist protection. Reduction in CDK5 activity corresponded to reduction in protein levels of CDK5 activator p35 during serum deprivation which was also reversed by μ-opioid receptor agonist. Phosphorylation of STAT3 at Serine 727 by CDK5 decreased during serum deprivation, and partly recovered by μ-opioid agonist. PI3K signaling pathway was not required for CDK5-mediated μ-opioid neuroprotection against serum deprivation. These findings indicate that neuroprotection by μ-opioid receptor agonist against serum deprivation is mediated by activation of CDK5 through up-regulation of p35 and phosphorylation of STAT3 by CDK5 may contribute to the neuroprotection.

Keywords: Cyclin-dependent kinase 5; p35; μ-Opioid agonist; Neuronal survival; Serum deprivation; Neuroprotection; p-STAT3; PI3K

Introduction

Opium, which consists of more than 20 alkaloids, is an extract of the exudates derived from seedpods of the opium poppy. It is widely used as a powerful analgesic. Morphine, one alkaloid working through μ-opioid receptor, is extensively used in clinical medicine. DAMGO, an enkephalin-derived peptide, which improves the selectivity to 100-fold for the μ receptor over other opioid receptors is commonly used as a tool in opioid study. In addition to well characterized effects on regulation of intestinal mobility and immune system, several report studies suggest that opioids elicit other biological effects independent of their analgesic properties (Gross et al., 2004; Lim et al., 2004; Jafari et al., 2004). Of major interest here, opioids have been implicated in determining cell survival and neuroprotection (Zohar et al., 2006; Lee et al., 2004; Lim et al., 2004; Iglesias et al., 2003).

Activation of the opioid receptors has been shown to activate the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway which has been linked neuroprotection (Iglesias et al., 2003). However, other reports suggest that the PI3K/Akt pathway is a feature of cell survival and not specific for neuroprotection. This is supported by the observation that neuroprotection by opioids persists for 24 h, whereas Akt phosphorylation is transient in the serum deprivation model of opioid neuroproteciton (Iglesias et al., 2003). Thus, there must be some other molecules in neurons that are important for opioid neuroprotection.

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A neuron-specific signaling molecule implicated in determination of neuronal survival and cell death is cyclin-dependent kinase 5 (CDK5). CDK5 is a unique member of the cyclin-dependent kinase family of CDKs, activation of CDK5 kinase activity requires association with its neuron-specific activators, p35 and p39; however, these cofactors, which are believed to provide substrate specificity, differ markedly from other CDK activating subunits called cyclins in both their primary sequence and regulation. Active CDK5 is essential for neuronal migration, neurite outgrowth and laminar configuration of the cerebral cortex during development (Lew et al., 1994; Tsai et al., 1994; Humbert et al., 2000), modulation of dopaminergic and glutamatergic transmission (Chergui et al., 2004), and endocytosis of synaptic vesicles (Tomizawa et al., 2003). However, overactivation of CDK5 is toxic to cells. Interestingly, increased CDK5 activity can be induced by proteolytic cleavage of p35 to p25 by the calcium activated proteases called calpain. Increased conversion of p35 to p25 causes prolonged activation, abnormal cellular location and substrate specificity changes of CDK5 leading to neurotoxicity, hyperphosphorylation of tau, cytoskeletal disruption and promotion of apoptosis in primary and cultured neurons (Kusakawa et al., 2000; Lee et al., 2000; Patrick et al., 1999). Conversely, a critical role for CDK5 in mouse survival has also been described. In cortical neurons, CDK5 has been reported to play a key role in promoting survival by negative regulation of c-Jun N-terminal kinase 3 (Li et al., 2002a,b) and Akt activity through the neuregulin/PI3K signaling pathway (Li et al., 2003). In myoblasts, overexpression of dominant-negative-Akt reduced CDK5 activity, indicating that CDK5 might be a downstream molecule of Akt. Taken together, these studies suggest that CDK5 contributes to cell survival or apoptosis depending on the type of cell and or stimuli in question (Cheung and Ip, 2004).

Since CDK5 has been implicated in survival, we hypothesized that CDK5 may play a role in neuroprotection. To address this hypothesis, we assessed the role of CDK5 in neuroprotection from serum deprivation via a selective μ-opioid receptor in differentiated SH-SY5Y cells and primary hippocampal cultures. Here we report that CDK5 activity is required for μ-opioid receptor agonist, DAMGO, to protect primary and cell line-derived neurons from serum deprivation independent of AKT signaling.

Material and methods

Cell culture and treatments

SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with and without 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in tissue culture dishes under a humidified incubator with 5% CO_2 at 37°C. For differentiation, the medium was replaced with fresh medium containing 10 μM *all-trans* retinoic acid (RA) the day after plating for 5 days (d) and changed every 2 d. RA differentiation of SH-SY5Y cells is associated with increased expression of μ-opioid receptor, as determined through binding experiments (Yu and Sadee, 1988; Zadina et al., 1994) and a constant cell number by [^{3}H]-thymidine incorporation assay (Iglesias et al., 2003; Datki et al., 2003), thus differentiated cells are both morphologically and physiologically very close to living neurons in the brain (Datki et al., 2003). For serum deprivation, adherent cells were rinsed three times with serum-free media and grown in serum-free media for 24 h or indicated time.

Primary culture of rat embryonic hippocampal neurons

Cultures of primary dissociated neurons were prepared from embryonic rat hippocampus as described (Buchhalter and Dichter, 1991). Pregnant Sprague–Dawley rats were narcotized with CO_2 for 90 s and then killed by cervical dislocation. The embryos (gestational days 18–19) were removed; the brains were dissected and placed on ice, and the hippocampi were dissected under microscopic visualization. The hippocampi were incubated for 20 min in DMEM (Invitrogen, Carlsbad, CA, USA) containing 0.03% trypsin (Sigma, St. Louis, MO, USA) at 37°C and 5% CO_2. They were then resuspended in growth medium containing DMEM, supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 10% Ham's F12 containing glutamine (Sigma, St. Louis, MO, USA) and 50 units/ml penicillin–streptomycin (Sigma, St. Louis, MO, USA). The cells were triturated with a sterile Pasteur pipette and plated onto poly(L-lysine) (Sigma, St. Louis, MO, USA) precoated 35-mm Petri dishes (Nunc) at a density of 1500–2000 cells/mm^2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated bovine calf serum (Hyclone, Logan, UT). After 7 d, cells were treated and harvested for further analysis.

Western blot analysis

Cells were harvested by washing in phosphate-buffered saline (PBS) and then scraping in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 20 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and protease inhibitor cocktail (Sigma, St. Louis, MO, USA)). The lysate was centrifuged at 12,000×*g* for 15 min at 4°C. Quantity of protein content in the supernatant was measured using a protein assay kit (Pierce, Tattenhall, UK). Protein extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a NC membrane (Amersham Biosciences Corp., NJ, and USA). The membrane was blocked with 5% non-fat dry milk in Tris buffered saline (pH 7.6) containing 0.1% tween-20 and was incubated with the following antibodies: rabbit anti-CDK5 (polyclonal, 1:250 dilution, Santa Cruz Biotechnology, CA, USA), mouse anti-p-ERK (monoclonal, 1:500 dilution, Santa Cruz Biotechnology, CA, USA), rabbit anti-PARP, rabbit anti-phospho-Serine-727-STAT3 and rabbit anti-total STAT3 (1:500 dilution, Cell Signaling Transduction, Beverly, MA, USA), rabbit anti-Akt (1:200 dilution, Santa Cruz Biotechnology, CA, USA) and monoclonal anti-p-Akt (1:500 dilution, Cell Signaling