Neuroscience Letters 450 (2009) 122–126

Contents lists available at ScienceDirect

Neuroscience Letters

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

Neuroprotection of interleukin-6 against NMDA attack and its signal transduction by JAK and MAPK

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ARTICLE INFO

Article history:
Received 21 August 2008
Received in revised form 2 November 2008
Accepted 22 November 2008

Keywords:
Interleukin-6
Neuroprotection
Cerebellar granule neurons
NMDA
JAK
MAPK

ABSTRACT

Cytokine interleukin-6 (IL-6) has been well shown to be elevated in brain injury and diseases. However, the significance of IL-6 production in such neuropathologic states remains controversial, and the intracellular signal-transduction pathways involved in the brain IL-6 action are primarily unclear. We previously indicated that exogenous IL-6 protected neurons against glutamate and N-methyl-D-aspartate (NMDA) attacks and the effects of IL-6 was blocked by anti-gp130 antibody. Here, we provide further evidence for the IL-6 neuroprotection and show signal molecules transducing the IL-6 message. The cerebellar granule neurons from postnatal 8-day infant rats were exposed to IL-6 for 8 days, and also pretreated chronically with Janus kinase (JAK) inhibitor AG490 and mitogen-activated protein kinase (MAPK) inhibitor PD98059. NMDA stimulated the cultured neurons for 30 min to induce neuronal injury and death. Cell counting kit-8 assay and Western blot were employed to measure neuronal vitality and cleaved caspase-3 expression, respectively. The chronic IL-6 exposure prevented the suppression of the neuronal vitality and the enhancement of the cleaved caspase-3 level induced by NMDA. The neuroprotective effect of IL-6 depended on IL-6 concentration and neuronal damaged degree. IL-6-induced STAT3 phosphorylation was inhibited by AG490 but not by PD98059; and IL-6-induced ERK1/2 activation was blocked by PD98059 but not by AG490. Either AG490 or PD98059 blocked the IL-6 protection against the NMDA-elicited neuronal vitality decrease and caspase-3 activation increase. These findings suggest that IL-6 protects neurons from NMDA-induced excitoxicity and the IL-6 neuroprotection may be transduced by both JAK/STAT3 and RAS/MAPK pathways.

Proinflammatory cytokine interleukin-6 (IL-6), which is classically known to be mainly from immune and hematopoietic systems, is also expressed by neurons and glial cells in the central nervous system (CNS). The expression of IL-6 is enhanced in various chronic or acute CNS disorders, for example, brain injury, infections, ischemia, and multiple sclerosis, as well as Alzheimer and Parkinson diseases [1,2,5,8,21]. The elevated IL-6 level during the CNS injury and diseases must have its functional significance. However, the significance of IL-6 production in such neuropathologic states remains controversial. Some reports present that IL-6 is a neurotrophic cytokine expressed in both neurons and glia. IL-6 protects neurons against glutamate and N-methyl-D-aspartate (NMDA) excitotoxicity in vitro [3,21,28] and prevents the brain from ischemic or excitotoxic attacks in vivo [1,8,11,13,18,24]. Nevertheless, other studies represent that the endogenous or exogenous increase of CNS IL-6 contributes to the pathogenesis of neurodegenerative disorders [5,15,19–21]. Thus, understanding the dichotomy pathogenesis/neuroprotection of IL-6 may provide a rationale for better therapeutic strategies [14].

In the immune and hematopoietic systems, IL-6 is a pleiotropic cytokine and has multiple functions. Since injury to CNS elicits an inflammatory response involving activation of microglia, brain macrophages and astrocytes [17], the processes likely mediated by the release of proinflammatory cytokines including IL-6 are extremely complicated. Under our cultured conditions, we previously indicated that the chronic IL-6 exposure prevented the cerebellar granule neurons (CGNs) from the decrease in neuronal vitality, the increase in apoptotic neurons, and the overload of intracellular Ca^{2+} induced by glutamate and NMDA, suggesting the neuroprotective effect of IL-6 [16,26]. Here, we provide further evidence for the IL-6 neuroprotection.

Although the neuroprotection of IL-6 has been reported, the mechanisms through which IL-6 accomplishes the neuroprotective effect are less known. Interaction of IL-6 with target cells in the presence of membrane or soluble IL-6 receptor protein

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doi:10.1016/j.neulet.2008.11.051

promotes dimerization of gp130, a 130 kDa of intracellular signal-transduction glycoprotein. The dimerized gp130 triggers activation of Janus kinase (JAK) and in turn, phosphorylates the signal transducer and activator of transcription-3 (STAT3) that translocates into the nucleus where it activates transcription [6]. Besides activation of the JAK/STAT pathway, RAS/mitogen-activated protein kinase (MAPK) pathway is also activated in many tissues in response to IL-6 [6]. Several reports have indicated that the JAK/STAT pathway is involved in the effects of IL-6 on neuronal functional disorders [6,15,18]. The RAS/MAPK pathway, however, has not been clearly elucidated in the IL-6 neuroprotection. We previously showed that anti-gp130 antibody blocked the suppressive effects of IL-6 on the glutamate- and NMDA-elicited intracellular Ca^{2+} overload, and that the levels of phospho-STAT3 and phospho-ERK1/2 (extracellular signal-regulated kinases 1/2), a member of MAPK family, were significantly increased in the IL-6-pretreated neurons [16,26], implying that gp130-STAT3 and gp130-ERK pathways are related to IL-6 role in neurons. Thus, in the current study, we applied inhibitors of JAK and MAPK/ERK kinase (MEK) to prove the hypothesis that IL-6 may activate both the JAK/STAT and RAS/MAPK pathways to accomplish its neuroprotection.

Sprague–Dawley infant rats of postnatal day 8 (The Center of Experimental Animals, Nantong University, China) were anesthetized by hypothermia by placing them in an ice bath for 2–4 min. The cerebella were removed from the rats and minced with sterile surgical blades. The minced cerebella were chemically dissociated in the presence of trypsin (Amresco, USA) and DNase I (Worthington, USA) and plated in poly-L-lysine-coated (50 mg/L) wells. Cells were seeded at a density of 2.83×10^5 cells/cm^2 in the complete culture medium composed of basal Eagle's medium with Earle's salts (Sigma, USA) supplemented with 0.1 g/L gentamicin, 2.2 g/L $NaHCO_3$, 2.385 g/L HEPES, 25 mM KCl, and 10% heat-inactivated fetal bovine serum (Amresco, USA). The sample was incubated at 37 °C with a humidified 5% CO_2/95% air atmosphere in an incubator (ESPEC BNA-311, Japan). Cytosine arabinoside (10 μM) was added 24 h after the cell plating to inhibit glial proliferation, and thus CGNs developed under the cultured conditions.

Rat recombinant IL-6 (R&D Systems, USA) at a concentration of 10, 40 or 160 ng/mL was added to the cultures of CGNs on days 1, 4 and 7 in order to keep the concentration of exogenously added IL-6 relatively constant during the period of chronic treatment. JAK inhibitor AG490 (Calbiochem, USA) or MEK inhibitor PD98059 (Calbiochem, USA) was added to the cultures of CGNs every 36 h at a dose of 5 μM (AG490) or 2.5 μM (PD98059). The selection of the doses for AG490 and PD98059 was on the basis of the research reports [4,27] and our pre-experimental data. NMDA (Sigma, USA) was dissolved in Locke's solution containing 10 μM glycine, which was applied to the 8-day cultures at a concentration of 10, 100, 1000 or 10,000 μM for 30 min at 20 °C. The Locke's solution was then replaced by the complete culture medium. The cultures were again incubated in a humidified incubator containing 5% CO_2 at 37 °C for 18 h.

Neuronal vitality of the cultured CGNs that had been treated with various drugs described as above was measured by use of cell counting kit-8 (CCK-8) assay. CCK-8 (Dojindo Laboratories, Japan) solution (10 μL) was added to the cultures (100 μL) followed by the incubation with 5% CO_2 at 37 °C for 4 h. Their optical density (OD) was read on a Universal Microplate Reader (Elx 800, Bio-Tek instruments, Inc., USA) using a test wavelength of 450 nm.

For Western blot analysis, the cultured CGNs were homogenized in lysis buffer containing 250 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS. Immediately before use, 50 mM phenylmethanesulfonyl (Sigma, USA) and 1 mM dithiothreitol (Bio-Basic Inc., Canada) were added to the lysis buffer. The supernatant was collected by centrifuging at 12,000 × *g* for 15 min. Total cellular protein were mixed with sample buffer and boiled for 5 min. Then the cellular protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine difluoride membrane using a semi-dry transfer apparatus. After blocking nonspecific binding with 5% defatted milk, the membranes were probed for caspase-3 (1:200; Santa Cruz Biotechnology, USA), phospho-STAT3 (1:500; Cell Signaling Technology, USA) and phospho-ERK1/2 (1:750; Cell Signaling Technology, USA) at 4 °C overnight, and followed by incubation with a horseradish peroxidase-conjugated secondary antibody and visualization with ECL reagent. Blots were reprobed with β-actin antibody (1:5000; Sigma, USA). Then the chemiluminescence was exposed to X-ray film. Expression of β-actin was used as control for equal gel loading.

Data were presented as mean ± standard deviation ($M \pm$ S.D.). Statistical analysis was carried out with Stata software (Computer Resource Center 7.0, USA). The significance of the differences between the means was determined using the one-way analysis of variance (ANOVA), followed by Student–Newman–Keul's test to compare the data of all groups between each other. Statistical significance was set at $p < 0.05$.

Stimulation of the cultured CGNs with NMDA (10, 100, 1000 and 10,000 μM) elicited a notable decrease in OD values reflecting the neuronal vitality (Fig. 1A). The higher doses of NMDA (100, 1000 and 10,000 μM) induced a greater attenuation of neuronal vitality than the lower dose of NMDA (10 μM) did (Fig. 1A). The OD values of the cultured CGNs that were chronically exposed to IL-6 (10, 40 and 160 ng/mL) were elevated compared with that of the neurons lacking IL-6 pretreatment, and the effect was enhanced with the increase of IL-6 concentrations (Fig. 1B). After the CGNs were pretreated with IL-6 (10, 40 and 160 ng/mL), the stimulation with the lower dose of NMDA (10 μM) no longer induced the attenuation of neuronal vitality, but still produced a IL-6-dose-dependent increase in the neuronal vitality similar to those neurons without NMDA stimulation (Fig. 1B). Compared with the CGNs lacking IL-6 pretreatment, the IL-6-pretreated neurons also obtained an improvement in the neuronal vitality when they were stimulated with the higher dose of NMDA (100 μM), although the neuronal vitality did not reach the level before NMDA stimulation (Fig. 1B). The results show that the antagonizing effect of IL-6 on 10 μM of NMDA was stronger than that of IL-6 on 100 μM of NMDA.

NMDA (100 μM) stimulation of the cultured CGNs lacking the chronic IL-6 exposure evoked an increase in the expression of cleaved caspase-3 protein (Fig. 2). NMDA stimulation of the CGNs pretreated chronically with IL-6 (40 ng/mL) resulted in a significant decrease in the cleaved caspase-3 expression compared with those neurons lacking IL-6 pretreatment (Fig. 2). The IL-6 chronic exposure alone did not notably affect the apoptotic enzyme activation (Fig. 2).

After a chronic combined pretreatment of the CGNs with JAK inhibitor AG490 (5 μM) and IL-6 (40 ng/mL), the NMDA stimulation led to a notable reduction in the OD value relative to those CGNS lacking AG490 pretreatment, with a reversion to the level only with NMDA stimulation (Fig. 3). Similarly, MEK inhibitor PD98059 (2.5 μM) blocked the IL-6 prevention of the NMDA-induced inhibition of the neuronal vitality (Fig. 3). The combined pretreatment of the neurons with AG490 and PD98059 also blocked the IL-6 neuroprotection against the NMDA-induced vitality suppression (Fig. 3). The pretreatment with AG490 or/and PD98059 alone did not significantly influence the neuronal vitality in comparison with the control lacking any treatment (Fig. 3).

As exhibited in Fig. 2, NMDA caused the cultured CGNs to upregulate the cleaved caspase-3 expression, and IL-6 depressed the NMDA-induced enhancement of the caspase-3 activation (Fig. 4). Either AG490 or PD98059 reversed the effect of IL-6 inhibiting NMDA-evoked increase in the caspase-3 activation (Fig. 4).