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Hepatocyte growth factor improves synaptic localization of the NMDA receptor and intracellular signaling after excitotoxic injury in cultured hippocampal neurons

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Abstract

To examine the effects of HGF on synaptic densities under excitotoxic conditions, we investigated changes in the number of puncta detected by double immunostaining with NMDA receptor subunits and presynaptic markers in cultured hippocampal neurons. Exposure of hippocampal neurons to excitotoxic NMDA (100 μM) decreased the synaptic localization of NMDA receptor subunit NR2B, whereas synaptic NR1 and NR2A clusters were not altered. Colocalization of PSD-95, a scaffolding protein of the receptor, with the presynaptic protein synapsin I was also decreased after excitotoxicity. Treatment with HGF attenuated these decreases in number. The decrease in the levels of surface NR2B subunits following the addition of the excitotoxic NMDA was also attenuated by the HGF treatment. The decrease in CREB phosphorylation in response to depolarization-evoked NMDA receptor activation was prevented by the HGF treatment. These results suggest that HGF not only prevented neuronal cell death but also attenuated the decrease in synaptic localization of NMDA receptor subunits and prevented intracellular signaling through the NMDA receptor.

Keywords: Hepatocyte growth factor; Neuronal injury; *N*-methyl-D-aspartate receptor; PSD-95

Introduction

The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of ionotropic glutamate receptors, is highly permeable to Ca^{2+} and Na^{+} (Dale and Roberts, 1985) and plays a pivotal role in the regulation of neuronal development, learning and memory, and neurodegenerative diseases (Dingledine et al., 1999). NMDA receptors are heteromeric complexes of NR1 and 4 types of NR2 (NR2A-2D), or NR3 subunits (Das et al., 1998; Ishii et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991; Nakanishi, 1992; Nishi et al., 2001). Whereas NR1 is the principal subunit for the channel activity of the NMDA receptor, the NR2 subunits serve to modulate the properties of these heteromeric receptors (Hollmann and Heinemann, 1994).

The postsynaptic density (PSD), which underlies the postsynaptic membrane at excitatory synapses, has been implicated in the linkage of receptors to signaling proteins and to the cytoskeleton (Kennedy, 1997; Kim and Sheng, 2004; Klauck and Scott, 1995; Ziff, 1997). PSD-95, a major protein component of the PSD, interacts with NMDA receptor subunits NR2A and NR2B by binding between PDZ (postsynaptic density-95, PSD-95/Discs large, Dlg/zona occludens-1, ZO-1) domains of PSD-95 and the C-terminal PDZ-binding motif of the receptor proteins (Cho et al., 1992; Kim and Sheng, 2004). PSD-95 also binds to signaling proteins such as neuronal nitric oxide synthase (Brenman et al., 1996) and synaptic Ras-GTPase activating protein p135synGAP (Chen et al., 1998; Kim et al., 1998) and organizes these intracellular signaling complexes. Therefore, it has been implied that PSD-95 links the NMDA receptor to intracellular

Abbreviations: CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; CREB, cAMP-response-element-binding protein; HGF, hepatocyte growth factor; NMDA, *N*-methyl-D-aspartate; PI, propidium iodide; PSD, postsynaptic density.

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signaling pathways at the synapse and plays an important role in synaptic plasticity and learning. With regard to this, in mice carrying a targeted mutation in their PSD-95 gene, NMDA receptor-mediated synaptic plasticity was altered and spatial learning in a water maze was impaired (Migaud et al., 1998).

In contrast to the crucial roles of the NMDA receptor in physiological activities such as learning and memory, an excessive activation of the receptor has been associated with diverse neurological and neurodegenerative disorders, including cerebral ischemia, epilepsy, Parkinson's disease, Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis (Dingledine et al., 1999). Therefore, it has become an important objective to investigate strategies to protect cells from NMDA receptor-mediated excitotoxicity. The hepatocyte growth factor (HGF), which was found to be a potent mitogen for hepatocytes (Nakamura et al., 1984, 1989), acts as an organotrophic factor for regeneration and has a protective effect in various organs (Balkovetz and Lipschutz, 1999; Matsumoto and Nakamura, 1996; Matsumoto and Nakamura, 2001; Zarnegar and Michalopoulos, 1995). In addition, HGF is known to evoke diverse cellular responses, including motogenic, morphogenic, angiogenic, and anti-apoptotic activities in various types of cells (Matsumoto and Nakamura, 1996; Nakamura et al., 1989; Thompson et al., 2004; Zarnegar and Michalopoulos, 1995). In the central nervous system, HGF and its c-Met receptor were found to function in a variety of ways (Achim et al., 1997; Honda et al., 1995; Sun et al., 2002a,b), including protection of tyrosine hydroxylase-positive midbrain neurons, as well as hippocampal and cortical neurons, against aging-related cell death in culture (Hamanoue et al., 1996; Honda et al., 1995; Machide et al., 1998). We recently demonstrated that HGF prevented *in vivo* ischemic brain injuries (Date et al., 2004; Niimura et al., 2006). Furthermore, HGF improved learning and memory dysfunction of ischemic rats in our previous study (Date et al., 2004). Although HGF exerts protective effects on cultured hippocampal neurons under pathophysiological conditions (Ishihara et al., 2005), it is still not clear whether HGF affects synaptic function of neurons under such conditions. In the present study, to achieve further insight into the reason for the potency of HGF treatment, we examined the effect of HGF on synaptic clustering of NMDA receptor subunits and PSD-95 in hippocampal neurons after excitotoxic injury. To assess the biochemical response to excitatory input, we furthermore evaluated the phosphorylation of cAMP-response-element-binding protein (CREB) mediated by the NMDA receptor. The results obtained show that HGF not only prevented the decrease in the number of synaptic NMDA receptor subunits and PSD-95 but also attenuated the decrease in the surface expression of NDMA receptor subunits. Furthermore, HGF improved phosphorylation of CREB in response to depolarization-evoked activation of the NMDA receptor.

Experimental procedures

Primary hippocampal cell cultures

Primary hippocampal cell cultures were prepared from fetal rats at gestational day 18 as described previously (Huettner and Baughman, 1986), with slight modifications (Ishihara et al., 2005). Brains were dissected out and the pooled hippocampi were dissociated by incubation at 37 °C for 30 min in Hank's balanced salt solution containing 15 U/ml papain, 210 U/ml deoxyribonuclease I, 1 mM L-cysteine, and 0.5 mM EDTA. The dispersed cells were resuspended in Dulbecco's Modified Eagle's Medium containing 10% horse serum, and plated at a density of 40,000 cells/cm^2 on 12-well plates or in 35-mm dishes coated with poly-L-lysine. At 24 h after plating, the medium was replaced with serum-free Neurobasal medium containing 2% B27 supplement (Gibco-BRL, Rockville, MD, USA) and 0.5 mM glutamine. To inhibit proliferation of non-neuronal cells, we added cytosine arabinoside (1 μM) to each plate or dish. At 3 and 10 days *in vitro* (DIV), one-half of the medium was replaced with fresh Neurobasal medium having the 2% B27 supplement and 0.5 mM glutamine. Cultures were maintained at 37 °C in a 5% CO_2 incubator and used for experiments at 15–18 DIV.

For the experiment of high K^+-induced CREB phosphorylation, 10 μM 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), 10 μM nifedipine, and 0.3 μM TTX were added 30 min before treatment with 57 mM KCl to inhibit AMPA receptors, L-type calcium channels, and spontaneous synaptic activity, respectively. Hippocampal cells were depolarized for 3 min with 10 mM HEPES buffer, pH 7.4, containing 60 mM KCl, 67 mM NaCl, 2 mM $CaCl_2$–$2H_2O$, 10 mM D-glucose, 10 μM glycine, 10 μM CNQX, 10 μM nifedipine, and 0.3 μM TTX. To inhibit the NMDA receptor, we added 10 μM MK-801 to cultures in some experiments.

Recombinant HGF

Human recombinant HGF was purified from conditioned medium of Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA as described earlier (Nakamura et al., 1989). The purity of hrHGF was >98%, as determined by SDS-PAGE.

Cell viability assay

Hippocampal cells were washed twice with 10 mM HEPES buffer, pH 7.4, containing 144 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, and 10 mM D-glucose and were then incubated for 15 min at 37 °C in a 5% CO_2 incubator with 100 μM NMDA in 10 mM HEPES buffer, pH 7.4, containing 144 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 10 mM D-glucose, and 10 μM glycine. The hippocampal cells were then washed and maintained in Neurobasal medium containing 2% B27 supplement and 0.5 mM glutamine. After 1, 6 or 24 h of incubation, the cells were incubated with 2 μg/ml propidium iodide (PI) for 20 min. After having been washed with phosphate-buffered saline, cells were fixed in 4% paraformaldehyde to determine the total number of neurons by immunostaining with anti-microtubule-associated protein 2 (MAP-2) antibody. Fluorescent images of cells were captured by a CCD camera (DP50, Olympus, Tokyo, Japan) mounted on an Olympus BX52 microscope equipped with a mercury arc lamp. The number of PI- or MAP-2-positive cells was counted in 10 randomly