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Insulin-like growth factor-1 modulates Ca²⁺ homeostasis and apoptosis of cultured dorsal root ganglion neurons with excitotoxicity induced by glutamate

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Insulin-like growth factor-1 (IGF-1) is a neurotrophic factor and a potent anti-apoptotic factor. IGF-1 plays an important role in promoting axonal growth from dorsal root ganglion (DRG) neurons and prevents apoptosis in DRG neurons. Whether IGF-1 could modulate Ca²⁺ homeostasis and apoptosis of sensory DRG neurons with excitotoxicity induced by glutamate (Glu) is still unknown. In the present study, primary cultured DRG neurons were used to determine the effects of IGF-1 on Ca²⁺ homeostasis and apoptosis of sensory DRG neurons with excitotoxicity induced by Glu. Intracellular Ca²⁺ concentration ([Ca²⁺]_i) in isolated DRG neurons using the fluorescent Ca²⁺ indicator fura-3 was measured by confocal laser scanning microscope (CLSM). Procaspase-3 expression was detected by Western blot analysis. Application of 0.2 mmol/L Glu evoked an increase in [Ca²⁺]_i, confirming the excitatory effect of Glu at this stage. The decrease of procaspase-3 expression levels after application of 0.2 mmol/L Glu suggested the apoptotic effects of Glu. These effects could be inhibited by the presence of IGF-1. In conclusion, we demonstrated that IGF-1 could modulate Ca²⁺ homeostasis and apoptosis of sensory DRG neurons with excitotoxicity induced by Glu. Both Ca²⁺ homeostasis and caspase-3 processing were implicated as the underlying neuroprotective mechanisms of IGF-1.

1. Introduction

Insulin-like growth factor-1 (IGF-1) is a polypeptide growth factor with a variety of functions in both neuronal and non-neuronal cells (Zheng and Quirion 2006). IGF-1 has broad range of neuroprotective effects and is a therapeutic candidate for neurodegenerative disease (Xing et al. 2005; Zhong et al. 2005; Escartin et al. 2007). IGF-1 is a neurotrophic factor (Batchelor et al. 2003; Guan et al. 2004; Baker et al. 2005; Zhao et al. 2005) and a potent anti-apoptotic factor (Russo et al. 2004; Guan et al. 2005). IGF-1 is emerging as an important growth factor able to modulate the programmed cell death (PCD) pathway mediated by the cysteine-dependent aspartate proteases (caspases) (Russell et al. 1998). IGF-1 promoted neuronal survival by activating its tyrosine kinase receptor IGF-1R (Zhong et al. 2004; McCusker et al. 2006). IGF-1 and its receptor (IGF-1R) are expressed in small DRG neurons. IGF-1 supports to a population of predominantly nociceptive neurons which may contribute to neuropathic pain (Craner et al. 2002). IGF-1 plays an important role in promoting axonal growth from dorsal root ganglion (DRG) neurons (Akahori and Horie 1997; Bomze et al. 2001; Jones et al. 2003).

Glutamate (Glu) is an excitatory amino acid (Yamada et al. 2005; Edling et al. 2007) and induces neuronal excitotoxicity by

activating N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptors to stimulate Ca²⁺ influx (Sanelli et al. 2007). Ca²⁺ is a universal second messenger that is a key component of myriad processes in all cell types. Perturbations in normal intracellular Ca²⁺ concentrations underlie many common pathological conditions (Montell 2005). Persistent elevation of intracellular Ca²⁺ concentration leads to apoptosis and cell destruction (Wu et al. 2004). Ca²⁺ signals play a role in the development and maturation of rat DRG neurons (Utzschneider et al. 1994).

It has been demonstrated that IGF-1 had the unique ability to sustain activation of Akt in the oligodendrocyte progenitor and provide long-term protection of these cells from Glu-mediated apoptosis (Ness et al. 2004). IGF-1 has effects on the cytotoxicity and apoptosis induced by okadaic acid (OA) in SH-SY5Y cells (Xing et al. 2005). It has been shown that IGF-1 promotes the survival of motor neurons (Bilak and Kuncl 2001; Vincent et al. 2004a) and prevents glutamate-induced motor neuron programmed cell death (Vincent et al. 2004b). Whether IGF-1 could modulate Ca²⁺ homeostasis and apoptosis of sensory DRG neurons with excitotoxicity induced by glutamate is still unknown. In the present study, primary cultured DRG neurons were used to determine the effects of IGF-1 on Ca²⁺ homeostasis and apoptosis of sensory DRG neurons with excitotoxicity induced by Glu.

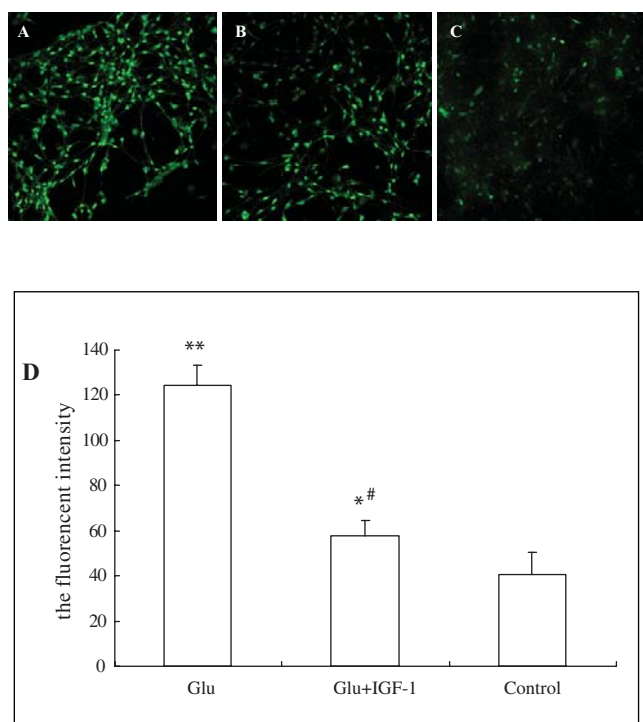


Fig. 1: CLSM photomicrographs of primary cultured DRG neurons at 48 hours of culture age then treated at different conditions for another 8 hours. Effects of IGF-1 on intracellular $[Ca^{2+}]_i$ in Glu incubated DRG neurons. Panel A: Glu (0.2 mmol/L). Panel B: Glu (0.2 mmol/L) plus IGF-1 (10 nmol/L). Panel C: Control. Panel D: Quantitative data of fluorescence intensity of DRG neuron preloaded with Fluo-3 AM. Bar graphs with error bars represent mean \pm SD (n=5). * $P < 0.01$ vs. Control, ** $P < 0.001$ vs. Control, # $P < 0.001$ vs. Glu

2. Investigations and results

2.1. Intracellular $[Ca^{2+}]_i$

The primary cultured DRG neurons at 48 h of culture age were treated with different agents for additional 8 h and then intracellular $[Ca^{2+}]_i$ was measured by CLSM. The Fluo-3 AM fluorescent intensity in control neurons (40.52 ± 10.06) was lower than in Glu incubated neurons (124.37 ± 8.69) ($P < 0.001$) or Glu plus IGF-1 incubated neurons (57.82 ± 6.73) ($P < 0.01$). The Fluo-3 AM fluorescent intensity in Glu plus IGF-1 incubated neurons was lower than that in Glu incubated neurons ($P < 0.001$) (Fig. 1).

2.2. Procaspase-3 expression

The effects of IGF-1 on procaspase-3 expression in Glu incubated DRG neurons were investigated by Western blot. Procaspase-3 expression in cultured DRG neurons was decreased after 8 h incubation with Glu (procaspase-3/ β -actin = 0.3413 ± 0.0632) as compared with control (procaspase-3/ β -actin = 0.7745 ± 0.0469) ($P < 0.001$). The decrease of procaspase-3 expression levels with application of Glu could be reversed by the presence of IGF-1 (procaspase-3/ β -actin = 0.6263 ± 0.0447) ($P < 0.001$). Decreased expression of procaspase-3 indicates increased processing of caspase that may be associated with apoptotic cell death (Fig. 2).

3. Discussion

Glu, the major excitatory neurotransmitter, can cause the death of neurons by a mechanism known as excitotoxicity. This is a calcium-dependent process and activation of the NMDA receptor subtype contributes mainly to neuronal damage, due to its high permeability to calcium (Del Río et al. 2008). Glu causes

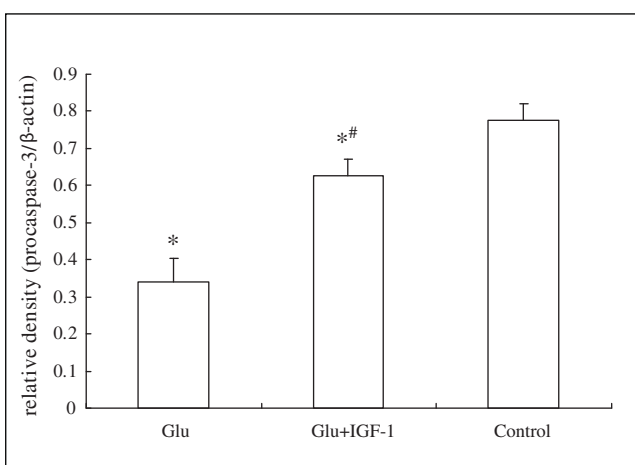
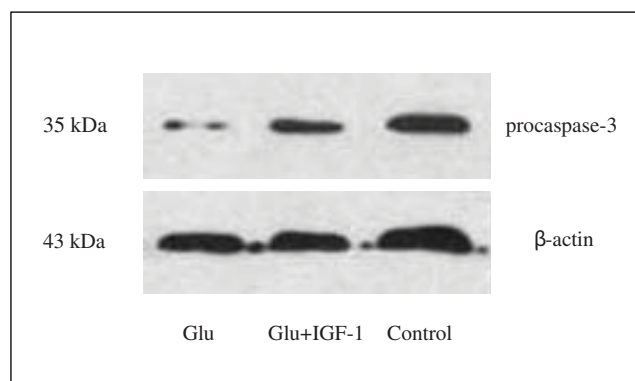


Fig. 2: Effects of IGF-1 on procaspase-3 expression in Glu incubated DRG neurons were investigated by Western blot. The relative density values of procaspase-3 expression are as follows: DRG cells were treated with Glu (0.3413 ± 0.0632). DRG cells were treated with Glu plus IGF-1 (0.6263 ± 0.0447). DRG cells were cultured continuously in growth media as control (0.7745 ± 0.0469). Bar graphs with error bars represent mean \pm SD (n=5). * $P < 0.001$ vs. Control, # $P < 0.001$ vs. Glu

apoptotic neuronal cell death at high concentrations (Zhang and Bhavnani 2006). In the present study, we found that: (1) the elevation of intracellular Ca^{2+} concentration induced by Glu could be inhibited by application of IGF-1; (2) the decrease of procaspase-3 expression levels after application of Glu could be reversed by the presence of IGF-1.

Glu can cause a slowly activating and reversible mobilisation of $[Ca^{2+}]_i$ in sensory neurons by activation of ionotropic receptors, and can induce oscillatory calcium transients by selectively activating metabotropic Glu receptors that are likely to be of the metabotropic Glu receptor (mGluR) 5 subtype (Crawford et al. 2000). N-Methyl-D-aspartate receptors (NMDAR) expressed on the central and peripheral terminals of primary afferent neurons are involved in nociception. Activation of NMDARs in DRG neurons caused a significant increase in intracellular $[Ca^{2+}]_i$ (Li et al. 2004). Activation of NMDARs on cultured DRG neurons sensitize voltage-dependent L-type Ca^{2+} channels which contribute to mechanically induced $[Ca^{2+}]_i$ transients through a protein kinase C (PKC)-mediated process (Chaban et al. 2004). It has been suggested that the elevation of intracellular $[Ca^{2+}]_i$ is association with desensitization of transient receptor potential vanilloid receptor 1 (TRPV1), previously called vanilloid receptor 1 (VR1) (Liu et al. 2003; van der Stelt et al. 2005; Mandadi et al. 2006; Xing et al. 2006; Yang et al. 2008). Changing of intracellular Ca^{2+} buffering affects the excitability of cultured DRG neurons. Increases in $[Ca^{2+}]_i$ caused membrane depolarization, altered the characteristics of evoked action potentials and activated potassium, chloride and non-selective

cation conductances (Ayar et al. 1999). In the present study, Glu (0.2 mmol/L) causes cytoplasmic Ca^{2+} overloading that could be reversed by application of exogenous IGF-1. This result confirms the neuroprotective actions of IGF-1 and suggests that it may modulate Ca^{2+} homeostasis.

Glu induces losses of oligodendrocytes and neurons and activation of caspase-3 in the rat spinal cord (Xu et al. 2008). Glu toxicity is a major contributor to death of oligodendroglia in diverse CNS disorders (Ness and Wood 2002). Glu induces caspase-3 activation and death of the late oligodendrocyte progenitor via activation of the AMPA/kainate Glu receptors (Ness et al. 2004). The activation of apoptosis is inversely correlated with $[\text{Ca}^{2+}]_i$ in cultured embryonic DRG neurons (Tong et al. 1997). DRG neurons apoptosis induced by Glu was dependent on the activation of pro-apoptotic protein caspase-3. Decreased expression of procaspase-3 induced by Glu indicates increased processing of caspase that may be associated with apoptotic cell death (Erhardt et al. 2001). Procaspase-3 expression assays were performed and supported the notion that IGF-1 could rescue apoptotic sensory DRG neurons with excitotoxicity induced by Glu. This result is consistent with that IGF-1 inhibited cerebellar granule neurons die of apoptosis and promoted neuronal survival via inhibiting caspase-3 activation (Zhong et al. 2004). It has been noted that modulation of ionotropic Glu receptors are involved in the regulatory actions of IGF-1 on neuronal plasticity (Gonzalez de la Vega et al. 2001). It has been suggested that IGF-1 prevents apoptosis in DRG neurons by regulating PI3K/Akt pathway effectors and by blocking caspase activation (Leininger et al. 2004). Several studies indicated that apoptotic neuronal death induced by excitotoxicity by administration of Glu appears to be mediated through the p38 signaling pathway in neurons (Nath et al. 2001; Chaparro-Huerta et al. 2005; Segura Torres et al. 2006; Chaparro-Huerta et al. 2008). One possible mechanism that IGF-1 plays an important regulatory function on preventing apoptosis of DRG neurons by modulating Ca^{2+} homeostasis and caspase-3 activity of DRG neurons with excitotoxicity induced by Glu.

Neurotrophins (nerve growth factor, NGF; brain-derived neurotrophic factor, BDNF) and monosialoganglioside (GM1) protect neurons against Glu excitotoxicity (Almeida et al. 2005; Huang et al. 2007). In the present study, IGF-1 also has this effect that it could modulate Ca^{2+} homeostasis and apoptosis of sensory DRG neurons with excitotoxicity induced by Glu. Both Ca^{2+} homeostasis and caspase-3 processing were implicated as the underlying neuroprotective mechanisms. The signaling pathway of the effects of IGF-1 on sensory DRG neurons with excitotoxicity induced by Glu remains to be investigated.

4. Experimental

4.1. DRG cell culture preparations

DRG was dissected out from embryonic 15-day-old Wistar rats. The animals were obtained from the Experimental Animal Center of Shandong University of China. DRG prior to establishment in culture was digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37 °C for 10 min and centrifuged for 5 min at 1×10^3 rpm. The supernatants were removed and the pellets were resuspended in Dulbecco's Modified Eagle Medium with F-12 supplement (DMEM/F-12) media (Gibco) and triturated using a sterile modified Pasteur's glass pipette. Cells were then filtered using a 130 μm filter followed by counting. Dissociated DRG cells were then cultured in 24-well clusters (Costar, Corning, NY, USA) for monitoring intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) under a confocal laser scanning microscope (CLSM) or flasks (Costar, Corning, NY, USA) for detecting procaspase-3 expression by Western blot analysis. DRG cells were plated at 1×10^5 cells/well for 24-well clusters which would contain a coverslip precoated with poly-L-lysine in each well and at a density of 5×10^5 cells/ml for flasks precoated with poly-L-lysine. Then DRG cells were cultured in culture media at 37 °C with 5% CO_2 for 24 h and then maintained in culture media containing cytarabine (ara-C) (5 $\mu\text{g}/\text{ml}$) for another 24 h to inhibit growth of non-neuronal cells,

and then cultured in culture media for another 8 h before observation. The composition of the culture media is D-MEM/F-12 (1:1) supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), insulin (0.25 $\mu\text{g}/\text{ml}$, Sigma), L-glutamine (0.1 mg/ml, Sigma), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$).

4.2. Exposure of IGF-1 on DRG neurons

At 48 h of culture age, all DRG neurons both in clusters and in flasks were exposed to Glu (0.2 mmol/L, Sigma), Glu (0.2 mmol/L) plus IGF-1 (10 nmol/L, Peptrotech). DRG cells were continuously exposed to growth media as control. Then all above cultures were incubated at 37 °C in a humidified 5% CO_2 -air atmosphere.

4.3. Intracellular $[\text{Ca}^{2+}]_i$ measurement

After 8 h exposure of Glu in the presence or absence of IGF-1, the DRG cells were loaded with fluo-3 acetoxymethyl ester (Fluo-3 AM, 5 $\mu\text{mol}/\text{L}$, Dojindo) for 30 min at room temperature. Excess dye was removed by washing with 0.01 mol/L PBS. The cells were imaged in 0.01 mol/L PBS. $[\text{Ca}^{2+}]_i$ was assessed by a CLSM (Leica). Fluo-3 AM was excited with the 488 nm of an argon ion laser, and the emitted fluorescence was measured at 510–530 nm. Each sample was randomly selected one visual field under the CLSM. All the DRG cells on the screen were monitored. By applying the computer software Image-Pro Plus 5.1 the intensities of the intracellular fluorescence were measured.

4.4. Western blot analysis for procaspase-3 expression

Procaspase-3 expression was analyzed by Western blot. Fresh cultured DRG neurons 8 h after treatment with different agents were homogenized in 10 mmol/L Tris homogenization buffer (pH 7.4) with protease inhibitors (Amersco). The samples were centrifuged at 12,000 rpm for 20 min and the supernatant collected for Western blot. After determining the protein concentrations of the supernatants (BCA method, standard: BSA), 50 μg protein of each sample was loaded onto the 12% SDS gel, separated by electrophoresis and transferred to PVDF membrane. The membranes were blocked in blocking buffer (5% nonfat milk) for 2 h at room temperature, and then were incubated with rabbit anti-procaspase-3 polyclonal IgG (1:1000, Cell signaling Technology) or mouse anti- β -actin monoclonal IgG (1:1000, Santa Cruz Biotechnology) overnight at 4 °C. After being washed three times for 10 min with washing solution, the membranes were incubated with goat anti-rabbit IgG-HRP (1:5000, abcam) or goat anti-mouse IgG-HRP (1:2000, Biotechnology). The immunoreactive bands were visualized by an ECL Western blotting detection kit (Pierce Biotech) on light sensitive film. The films were scanned and the images were analyzed quantitatively by using an ImagJ 1.39u image analysis software. The levels of procaspase-3 were expressed as the ratio of the protein to β -actin.

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