Regulatory effects of insulin-like growth factor-1 on the expression of sensory neuropeptide mRNAs in cultured dorsal root ganglion neurons with excitotoxicity induced by glutamate

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Abstract: Objective To determine the effects of insulin-like growth factor-1 (IGF-1) on the expression of preprotachykinin (PPT) mRNA encoding substance P (SP) and calcitonin gene-related peptide (CGRP) mRNA in cultured dorsal root ganglion (DRG) neurons with excitotoxicity induced by glutamate (Glu). **Methods** DRGs were dissected from embryonic day 15 Wistar rats. DRG neurons were dissociated and cultured for 48 h and then exposed to Glu (0.2 mmol/L) or Glu (0.2 mmol/L) plus IGF-1 (5 nmol/L, 10 nmol/L and 20 nmol/L) for 12 h. The DRG neurons in control group were exposed to only growth media throughout the experiment. After that, the living DRG neurons were observed under inverted phase contrast microscope and microphotographs were taken. The expression levels of PPT and CGRP mRNAs were detected by reverse transcription-polymerase chain reaction (RT-PCR). **Results** IGF-1 could inhibit Glu-induced shortening of neurite. Besides, IGF-1 could significantly increase the levels of PPT mRNA and CGRP mRNA in primary cultured DRG neurons with Glu-induced excitotoxicity, in a dose-dependent manner. **Conclusion** IGF-1 may exert neuroprotective effects on DRG neurons against Glu-induced excitotoxicity, probably through regulating the expression levels of PPT and CGRP mRNAs.

Keywords: insulin-like growth factor-1; glutamate; substance P; calcitonin gene-related peptide; dorsal root ganglion

1 Introduction

Insulin-like growth factor-1 (IGF-1) is a polypeptide growth factor with various functions in both neuronal and non-neuronal cells^[1]. IGF-1 can promote neuronal survival by activating its tyrosine kinase receptor IGF-1R^[2,3]. IGF-1 and IGF-1R are both expressed in small dorsal root ganglion (DRG) neurons. IGF-1 supports a population of predominantly nociceptive neurons that may contribute to neuropathic pain^[4]. IGF-1 also plays an important role in promoting axonal growth

from DRG neurons^[5-7].

Glutamate (Glu) is an excitatory amino acid^[8,9] 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^[10]. There has been convincing evidence that excessive extracellular accumulation of Glu is toxic to the central^[11] and the peripheral neurons^[12]. IGF-1 is a neurotrophic^[13-16] and potent anti-apoptotic factor^[17,18]. Substance P (SP) and calcitonin gene-related peptide (CGRP) are both sensory neuropeptides and are expressed in primary sensory DRG neurons^[19,20]. However, it is still unclear whether IGF-1 could regulate the expression of preprotachykinin (PPT) mRNA encoding SP and CGRP mRNA in DRG neurons with Glu-

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induced excitotoxicity. In the present study, by employing primary cultured DRG neurons, the protective effects of IGF-1 on the expression of PPT mRNA encoding SP and CGRP mRNA against Glu-induced excitotoxicity were investigated.

2 Materials and methods

2.1 DRG neuron culture DRGs were dissected from embryonic day 15 Wistar rats (Experimental Animal Center of Shandong University, China). Prior to culture, DRGs were digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37 °C for 10 min, and then centrifuged and triturated in growth media supplemented with 2.5% fetal bovine serum (Gibco). Then the dissociated DRG cells were plated at a density of 5×10^5 cells/mL in flasks (Costar, Corning, NY, USA) pre-coated with poly-L-lysine. Cells were cultured in DMEM/F12, supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), 0.25 µg/mL insulin (Sigma), 0.1 mg/mL L-glutamine (Sigma), 100 U/mL penicillin, and 100 µg/mL streptomycin. DRG cells were cultured at 37 °C with 5% CO₂ for 24 h and then maintained in culture media containing cytarabine (ara-C) (5 µg/mL) for another 24 h to inhibit the growth of non-neuronal cells.

2.2 Treatment of DRG neurons with Glu and IGF-1 After ara-C treatment, DRG neurons in flasks were exposed to Glu (0.2 mmol/L, Sigma), or both Glu (0.2 mmol/L) and IGF-1 (5 nmol/L, 10 nmol/L or 20 nmol/L; Peprotech) for an additional 12 h, respectively. In the control group, the DRG neurons were continuously exposed to only growth media. At 12 h of treatment, the neurons were observed under inverted phase contrast microscope and the microphotographs were taken.

2.3 Reverse transcription-polymerase chain reaction (RT-PCR) The expression levels of PPT mRNA encoding SP and CGRP mRNA were analyzed by RT-PCR after 12-h treatment with Glu or Glu plus IGF-1. β -Actin served as an internal control. Total RNA was extracted from each flask of cells using TRIzol (Gibco). mRNA level was analyzed by RT-PCR according to the methods described previously^[21,22]. cDNA was generated using M-MLV Reverse Transcriptase. The gene-specific primers were designed according to the published cDNA sequences and synthesized: PPT forward 5'-GCC CTT TGA GCA TCT TCT TC-3', reverse 5'-GTC TGA

GGAGGT CACCACAT-3', CGRP forward 5'-AGG TCG GGA GGT GTG GTG AA-3', reverse 5'-ATC CGC TTG AGG TTT AGC AGA G-3', β -actin forward 5'-ATC ATG TTT GAG ACC TTC AAC-3', reverse 5'-CAT CTC TTG CTC GAA GTC CA-3'. The predicted sizes of the amplified PPT, CGRP, and β -actin DNA products were 450 bp, 338 bp, and 317 bp, respectively.

PCR was performed for 35 cycles, including denaturation at 94 °C for 45 s, annealing at 58 °C for 60 s, and extension at 72 °C for 45 s. PCR was performed within the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products.

The amplified products were analyzed by standard agarose gel electrophoresis. Bands of cDNA were stained with ethidium bromide and visualized by a UV transilluminator. Photographs were taken and quantitative analysis was performed by using ImageJ analysis software. The levels of PPT and CGRP mRNAs were expressed as the ratio to that of β -actin.

2.4 Statistical analysis Data were expressed as mean $\pm$ SD, and analyzed by one-way analysis of variance (ANOVA) followed by SPSS software. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Alterations in neurite length after treatment with Glu or Glu plus IGF-1 As shown in Fig. 1, the length of neurite was decreased after Glu treatment, as compared with that in control group. However, this decrease could be reversed by treatment with different concentrations of IGF-1, to different extents.

3.2 Effect of IGF-1 on PPT mRNA expression As shown in Fig. 2, the ratios of PPT mRNA level to that of β -actin in Glu, Glu plus IGF-1 (5 nmol/L), Glu plus IGF-1 (10 nmol/L), Glu plus IGF-1 (20 nmol/L), and control groups were (0.5160 ± 0.0559) , (0.6050 ± 0.0577) , (0.7048 ± 0.0468) , (0.7745 ± 0.0541) , and (0.9195 ± 0.0390) , respectively. IGF-1 treatment could significantly attenuate the Glu-induced decrease in PPT mRNA expression level. Moreover, there were also significant differences among the Glu plus IGF-1 groups, indicating a dose-dependent effect of IGF-1.

Fig. 1 An observation of living DRG neurons after treatment with Glu or Glu plus IGF-1. IGF-1 could reverse the Glu-induced decrease of neurite length in DRG neurons. A: DRG neurons were exposed to Glu (0.2 mmol/L) for 12 h. B: DRG neurons were exposed to Glu (0.2 mmol/L) plus IGF-1 (5 nmol/L) for 12 h. C: DRG neurons were exposed to Glu (0.2 mmol/L) plus IGF-1 (10 nmol/L) for 12 h. D: DRG neurons were exposed to Glu (0.2 mmol/L) plus IGF-1 (20 nmol/L) for 12 h. E: The control group. Thick arrow: neuronal cell body. Thin arrow: neurite. Scale bar: 25 μ m.

Fig. 2 Effects of IGF-1 on PPT mRNA expression in DRG neurons, as detected by RT-PCR. A: Assay of PPT mRNA level in different groups. B: A quantification of RT-PCR results. The Glu-induced decrease in PPT mRNA level was significantly reversed by IGF-1 treatment, although it was still lower than that in control group. Moreover, there were significant differences among the Glu plus IGF-1 groups, suggesting a dose-dependent effect of IGF-1. *** P < 0.001 vs control, # P < 0.05, ### P < 0.001 vs Glu group, \$\$ P < 0.01, \$\$\$ P < 0.001 vs Glu + IGF-1 (5 nmol/L) group, & P < 0.05 vs Glu + IGF-1 (10 nmol/L) group. n = 5 in each group.

3.3 Effect of IGF-1 on CGRP mRNA expression The ratios of CGRP mRNA level to β -actin mRNA level in Glu (0.2 mmol/L), Glu plus IGF-1 (5 nmol/L), Glu plus IGF-1 (10 nmol/L), Glu plus IGF-1 (20 nmol/L), and control groups were (0.5038 ± 0.0627), (0.5996 ± 0.0350), (0.6620 ± 0.0340), ($0.7344 \pm$

0.0187), and (0.8447 ± 0.0623), respectively. IGF-1 treatment could significantly attenuate the Glu-induced decrease in CGRP mRNA expression level. Moreover, there were significant differences among the Glu plus IGF-1 groups, suggesting a dose-dependent effect of IGF-1 (Fig. 3).

Fig. 3 Effects of IGF-1 on CGRP mRNA expression level in DRG neurons, as investigated by RT-PCR. **A:** Assay of CGRP mRNA level in different groups. **B:** A quantification of RT-PCR results. The Glu-induced decrease in CGRP mRNA level was significantly reversed by IGF-1 treatment, although it was still lower than that in control group. Moreover, there were significant differences among the Glu + IGF-1 groups, suggesting a dose-dependent effect of IGF-1. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control, $^{\#}P < 0.05$, $^{###}P < 0.001$ vs Glu group, $^{\$}P < 0.05$, $^{sss}P < 0.001$ vs Glu + IGF-1 (5 nmol/L) group, $^{\&}P < 0.05$ vs Glu + IGF-1 (10 nmol/L) group.

4 Discussion

As a major excitatory neurotransmitter, Glu can cause the death of neurons by a mechanism known as excitotoxicity. In the contrast, IGF-1 can produce a broad range of neuroprotective effects, and serves as a therapeutic candidate for neurodegenerative disease^[23-25]. Sensory neuropeptide expression and release may reflect the function of sensory neurons^[20,26]. In the present study, we find that IGF-1 can significantly reverse the Glu-induced decrease in the levels of PPT mRNA encoding SP and CGRP mRNA in primary cultured DRG neurons, in a dose-dependent way.

SP is an 11-amino acid peptide sensory neurotransmitter

synthesized in the primary sensory neurons and released upon appropriate stimulations (such as capsaicin and potassium). It plays an important role in nociception and inflammatory actions^[19,26]. SP is a member of the tachykinin family, and most members of the tachykinin family, including SP, are generated by alternative splicing of PPT gene^[27]. SP is found in sensory nerve innervating peripheral tissues^[19]. Apart from its role in nociception, SP has also been found to participate in inflammatory actions, such as vasodilation, plasma extravasation^[28], cytokine release, and recruitment and proliferation of inflammatory cells^[29]. Here, PPT mRNA expression was used as an indicator of the neuro-protective effects of IGF-1 on DRG neurons with Glu-induced

excitotoxicity. The results show that IGF-1 could increase the level of PPT mRNA in primary cultured DRG neurons with Glu-induced excitotoxicity, and interestingly, in a dose-dependent manner. However, the mechanism still needs to be investigated.

CGRP is a 37-amino acid peptide widely distributed in the central and the peripheral nervous systems, including the primary sensory neurons and its peripheral and central branches^[30]. CGRP is released upon appropriate stimulation and plays an important role in pain signal transmission by potentiating nociceptive signaling and pain-related behavior mediated by SP^[31]. CGRP is also known to produce proinflammatory effects through vasodilation and promotion of protein extravasation^[32,33]. In the present study, CGRP mRNA expression was also used as an indicator to reflect the neuroprotective effects of IGF-1 on DRG neurons with Glu-induced excitotoxicity. Results show that IGF-1 can also promote CGRP mRNA expression in a dose-dependent manner, although the underlying mechanism still needs to be clarified.

In vitro DRG neurons can share characteristics with *in vivo* nociceptors and are frequently used as a tool to study the ion channels that underlie the transduction of noxious stimuli into electrical activity during sensory processing^[34]. A variety of chemical and environmental stimuli which might cause pain *in vivo* can also stimulate the cultured DRG neurons^[35]. Here, the primary cultured DRG neurons were used as an experimental model to detect the mRNA levels of sensory neuropeptides. We find that with the increases in mRNA levels of sensory neuropeptides, the neurite length would increase accordingly.

Taken together, IGF-1 may produce neuroprotective effects through regulating the expression of PPT mRNA encoding SP and CGRP mRNA in DRG neurons with Glu-induced excitotoxicity. However, the underlying mechanisms still need to be further investigated.

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胰岛素样生长因子-1对背根神经节神经元感觉神经肽 mRNA 表达的调节作用

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摘要: 目的 利用背根神经节(dorsal root ganglion, DRG)神经元, 观察胰岛素样生长因子-1(insulin-like growth factor-1, IGF-1)对谷氨酸(Glu)神经毒性引起的编码P物质(substance P, SP)的前速激肽原(preprotachykinin, PPT) mRNA和降钙素基因相关肽(calcitonin gene-related peptide, CGRP) mRNA 表达下降的调节作用。方法 取 15 d 胎龄大鼠的 DRG 神经元, 分散培养 48 h 后, 在培养液中加入 Glu (0.2 mmol/L), 或同时加入不同浓度的 IGF-1 (5 nmol/L, 10 nmol/L, 或 20 nmol/L) 孵育 12 h, 利用倒置相差显微镜对神经元活细胞进行观察, 并用 RT-PCR 法检测神经元中 PPT 和 CGRP 的 mRNA 表达水平。对照组 DRG 神经元培养液中不含 Glu 和 IGF-1。结果 Glu 能引起神经元突起的缩短, 而 IGF-1 则显著减弱这一作用。此外, Glu 的神经毒性使得 DRG 神经元内 PPT 和 CGRP 的 mRNA 水平显著降低, 而 IGF-1 则能明显抑制这种降低, 且呈一定的浓度依赖性。结论 IGF-1 可能通过调节 PPT 和 CGRP 的 mRNA 表达水平, 对 DRG 神经元产生保护作用。

关键词: 胰岛素样生长因子-1; 谷氨酸; P 物质; 降钙素基因相关肽; 背根神经节