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Regulatory effect of nerve growth factor on release of substance P in cultured dorsal root ganglion neurons of rat

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Abstract: Objective To investigate the regulatory effects of nerve growth factor (NGF) on basal and capsaicin-induced release of neuropeptide substance P (SP) in primary cultured embryonic rat dorsal root ganglion (DRG) neurons. **Methods** DRGs were dissected from 15-day-old embryonic Wistar rats. DRG neurons were dissociated and cultured, and then exposed to different concentrations of NGF (10 ng/mL, 30 ng/mL, or 100 ng/mL) for 72 h. The neurons cultured in media without NGF served as control. RT-PCR were used for detecting the mRNAs of SP and vanilloid receptor 1 (VR1) in the DRG neurons. The SP basal and capsaicin (100 nmol/L)-induced release in the culture were measured by radioimmunoassay (RIA). **Results** SP mRNA and VR1 mRNA expression increased in primary cultured DRG neurons in a dose-dependent manner of NGF. Both basal release and capsaicin-evoked release of SP increased in NGF-treated DRG neurons compared with in control group. The capsaicin-evoked release of SP also increased in a dose-dependent manner of NGF. **Conclusion** NGF may promote both basal release and capsaicin-evoked release of SP. NGF might increase the sensitivity of nociceptors by increasing the SP mRNA or VR1 mRNA.

Keywords: nerve growth factor; dorsal root ganglion; capsaicin; vanilloid receptor 1; substance P

1 Introduction

Among many neurotrophic factors that act on sensory neurons, nerve growth factor (NGF) has been studied extensively[1]. NGF initially interested neurobiologists because of its effects on the survival, differentiation and maturation of developing nervous system. It is now clear that NGF functions throughout the animal life with a wide repertoire of actions[2]. In the past years, several lines of evidence converged to indicate that NGF participates in structural and functional plasticity of the dorsal root ganglion (DRG). NGF may induce the intracellular events such as mitochondrial transportation or accumulation at the regions of focal NGF stimulation, and intracellular Ca^{2+} homeostasis[3].

Recently, NGF has been suggested to exert an acute effect on nociceptive sensory neurons in addition to its trophic effect[4]. NGF produces sensitization of nociceptive responses[5] and increases the capsaicin sensitivity of primary sensory neurons[6,7]. The increased NGF level in the inflamed tissues[8] is associated with inflammatory hyperalgesia[9,10]. Capsaicin-induced neuropeptide release is also related to pain sensation. The capsaicin functions through activation of the vanilloid receptor 1 (VR1) on the plasma membrane of primary sensory neurons to cause peptidic neurotransmitter release[11,12].

Substance P (SP), one of peptidic neurotransmitters in primary sensory neurons, could activate the upper sensory neurons to transmit pain signal from peripheral to central nervous system. It is also involved in inflammatory responses[13]. However, the relationship between the inflammatory hyperalgesia contributed by NGF and the neuropeptide release induced by capsaicin in the course of inflammatory responses remains unclear. In the present study, we exposed dissociated DRG neurons to different concentrations of NGF and analyzed SP mRNA and VR1 mRNA levels by RT-PCR. The amount of SP releases before

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and after capsaicin stimulation was detected by radioimmunoassay (RIA).

2 Materials and methods

2.1 DRG cell culture DRGs were dissected from 15-day-old embryonic Wistar rats (the Experimental Animal Center of Shandong University of 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). The dissociated DRG neurons were cultured in 24-well clusters (Costar, Corning, NY, USA) for monitoring SP levels using RIA, or in flasks (Costar, Corning, NY, USA) for detecting SP mRNA and VR1 mRNA by RT-PCR. The clusters and flasks were pre-coated with poly-L-lysine prior to plating DRG neurons. DRG neurons were plated at a density of 1×10^5 cells/well in clusters and 5×10^5 cells/mL in flasks. All cultures were maintained in the DMEM/F12 supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), insulin (0.25 μg/mL, Sigma), L-glutamine (0.1 mg/mL, Sigma), penicillin (100 U/mL), and streptomycin (100 μg/mL). DRG neurons both in the clusters and the flasks were exposed to different concentrations of NGF (10 ng/mL, 30 ng/mL, or 100 ng/mL). The DRG neurons exposed to the growth media without NGF were set as normal control. All cultures were grown at 37 °C in a humidified 5% CO_2/95% air atmosphere for 72 h.

2.2 RT-PCR for detection of the mRNAs of SP and VR1 The mRNA levels of SP and VR1 in 72-h-old cultures were analyzed by RT-PCR, with β-actin as an internal control.

Total RNA of the DRG neurons in each flask was isolated by TRIzol (Gibco) and the mRNA level was analyzed by RT-PCR according to the methods described previously[14]. The gene-specific primers were synthesized by use of the published cDNA sequences for SP, VR1 and β-actin. The synthetic oligonucleotide primer sequences for SP, VR1 and β-actin were as follows: SP: sense 5'-GCC CTT TGA GCA TCT TCT TC-3' and antisense 5'-GTC TGA GGA GGT CAC CAC AT-3'; VR1: sense 5'-CTG ACG GCA AGG ATG ACT-3' and antisense 5'-CCT AAG CAG ACC ACC CAA-3'; and β-actin: sense 5'-ATC ATG TTT GAG ACC TTC AAC-3' and antisense 5'-CAT CTC TTG CTC GAA GTC CA-3'. The predicted sizes of the amplified SP, VR1 and β-actin DNA products were 450 bp, 372 bp and 317 bp, respectively.

The PCR cycle profile was denaturation at 94 °C for 45 s, anneal at 58 °C for 45 s, and extension at 72 °C for 60 s. PCR was performed within the range of a linear correlation between the cDNA amount and the yield of PCR products.

The amplified products were analyzed by standard agarose gel electrophoresis and stained with ethidium bromide, and then visualized with a UV transilluminator (Jiangsu JEDA, JD801, China) and photographed. The photographs were analyzed quantitatively by a TotalLab image analysis software (Nonlinear Dynamics, UK).

2.3 RIA analysis for SP release After 72-h incubation, DRG neuron cultures were washed with release buffer (Hank's balanced salt solution supplemented with 10.9 mmol/L HEPES, 4.2 mmol/L sodium bicarbonate, 10 mmol/L dextrose, and 0.1% bovine serum albumin, pH 7.4) and then incubated in release buffer for 10 min at 37 °C for measuring the basal release of SP. Fresh release buffer containing the capsaicin (100 nmol/L) was added and incubated for an additional 10 min to detect the capsaicin-evoked release of SP. After each of incubation, the culture media were removed to measure the release of SP from the DRG neurons by RIA.

The RIA technique for measuring SP was similar to that reported previously[15]. The samples were reconstituted in PBS. Standards of synthetic SP (rat amino acid sequence) ranging 2.5 – 1 280 pg/assay tube were separately dissolved in 0.2 mL of PBS. The dissolved SP was then incubated with 0.1 mL of anti-SP antibody (anti-rat SP antibody) for 24 h at 4 °C. The anti-SP antibody cross-reacts 100% with rat SP, has < 0.01% cross-reactivity with neuropeptide K and neurokinin A, and has 0% cross-reactivity with neurokinin B and somatostatin (data from Department of Neurobiology, Second Military Medical University, China). The mixture was then incubated with 0.1 mL of ^{125}I-labeled SP (20 000 counts·min^{-1}/tube) in PBS for an additional 24 h at 4 °C. Free and bound neuropeptides were separated by incubation with 0.5 mL separating agent for 45 min. The RIA test tubes were centrifuged at 4 000 r/min (4 °C, 20 min). After removal of the supernatant fraction, the remaining ^{125}I in RIA test tubes were counted.

2.4 Statistical analysis Data were expressed as mean ± SD. Statistical significance was evaluated by one-way ANOVA followed by SPSS software. $P < 0.05$ was considered to be statistical significant.

3 Results

3.1 Effects of NGF on the mRNA expression of SP and

VR1 The ratios of SP mRNA/β-actin mRNA in 10 ng/mL, 30 ng/mL, and 100 ng/mL of NGF treated DRG neurons were 0.3633±0.0340, 0.5177±0.1057, and 0.8381±0.0641, respectively, all increased significantly compared with control group (0.1673±0.0140, $P<0.01$). The ratios of VR1 mRNA/β-actin mRNA in 10 ng/mL, 30 ng/mL, and 100 ng/mL of NGF treated DRG neurons were 0.1809 ± 0.0407, 0.2323 ± 0.0423, and 0.3211 ± 0.0394, respectively, also increased significantly compared with the control group (0.1037 ± 0.0164, $P < 0.01$). The differences between each dose group were significant ($P < 0.05$ or $P < 0.01$), indicating that NGF promoted the expressions of SP mRNA and VR1 mRNA in a dose-dependent manner in primary cultured DRG neurons (Fig. 1, 2).

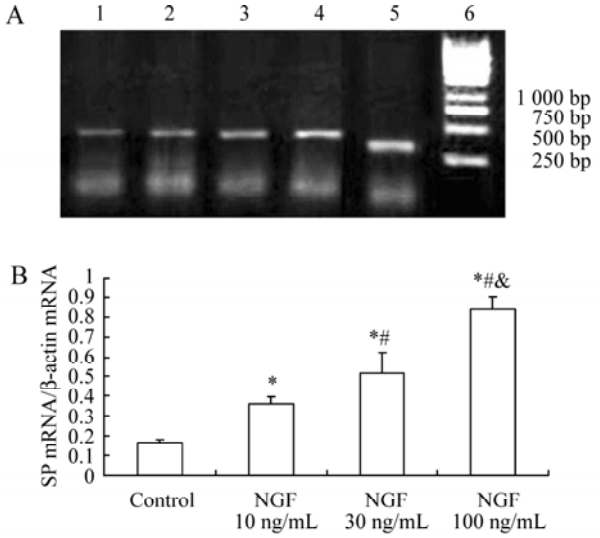

Fig. 1 Effects of different concentrations of NGF on the SP mRNA expression in cultured DRG neurons. A: Lane 1, Control; Lane 2, NGF 10 ng/mL; Lane 3, NGF 30 ng/mL; Lane 4, NGF 100 ng/mL; Lane 5, Internal control; Lane 6, DNA Marker. B: The quantitative analysis of the results of SP mRNA expression levels. Bar graphs with error bars represent mean ± SD ($n = 5$). $^{*}P < 0.001$ *vs* control, $^{\#}P < 0.001$ *vs* NGF 10 ng/mL, $^{\&}P < 0.001$ *vs* NGF 30 ng/mL.

3.2 NGF increased the basal and capsaicin-evoked SP release

In NGF-treated DRG neurons, both of SP basal release and capsaicin-evoked SP release significantly increased compared with control group. The SP basal release in 10 ng/mL, 30 ng/mL, and 100 ng/mL of NGF treated DRG neurons were 4.982±1.4 pg/well, 6.15±1.28 pg/well, and 7.148±1.13 pg/well, respectively, all significantly higher than the control group (3.09±1.21 pg/well, $P < 0.01$ or $P < 0.001$). The Capsaicin-evoked SP release in NGF 10 ng/mL, 30 ng/mL, and 100 ng/mL treated DRG neurons were 21.06±3.43 pg/well, 27.5±4.38 pg/well, and 33.54±2.77 pg/well, respectively, higher than the control group (13.43 ± 2.19 pg/well, $P < 0.01$ or $P < 0.001$) as well. Furthermore, NGF increased the capsaicin-evoked SP release in a dose-dependent manner ($P < 0.1$ or $P < 0.01$) (Fig. 3).

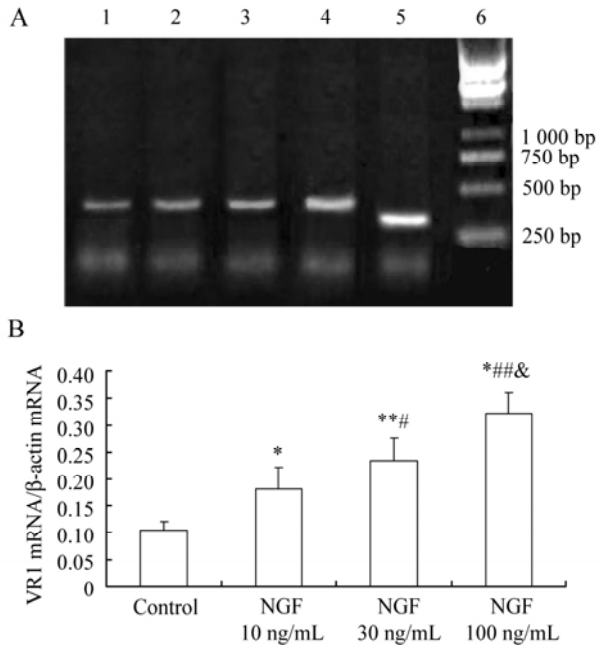

Fig. 2 Effects of different concentrations of NGF on the VR1 mRNA expression in cultured DRG neurons. A: Lane 1: Control; Lane 2: NGF 10 ng/mL; Lane 3: NGF 30 ng/mL; Lane 4: NGF 100 ng/mL; Lane 5: Internal control; Lane 6: DNA Marker. B: The quantitative analysis of the results of VR1 mRNA expression levels. Bar graphs with error bars represent mean ± SD ($n = 5$). $^{*}P < 0.01$ *vs* control, $^{**}P < 0.001$ *vs* control; $^{\#}P < 0.05$ *vs* NGF 10 ng/mL, $^{\#\#}P < 0.01$ *vs* NGF 10 ng/mL; $^{\&}P < 0.01$ *vs* NGF 30 ng/mL.

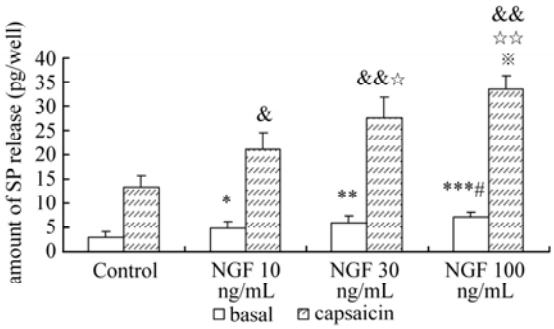

Fig. 3 Radioimmunoassay analysis of the amount of basal SP release or capsaicin-evoked SP release from dissociated cultured DRG neurons of 72-h culture age in the presence or absence of NGF. $n = 5$ in each group. Basal release: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control; $^{\#}P < 0.05$ *vs* NGF 10 ng/mL. Capsaicin-evoked release: $^{\&}P < 0.01$, $^{\&\&}P < 0.001$ *vs* control; $^{\star}P < 0.01$ *vs* NGF 10 ng/mL, $^{\star\star}P < 0.001$ *vs* NGF 10 ng/mL; $^{※}P < 0.05$ *vs* NGF 30 ng/mL.

4 Discussion

The present study demonstrated that NGF promoted the capsaicin-evoked SP release in the primary cultured DRG neurons in a dose-dependent manner. The basal SP release was also increased in NGF treated cultures, which may reflect an increased synthesis of SP in DRG neurons. These results indicated that exogenous NGF could improve neuronal function status, since stimulus-evoked neuropeptide release is a key measure for sensory neuronal function[16].

NGF not only supports the survival, differentiation and maturation of neurons[17], but also plays an important role in nociceptive responses and inflammatory actions of sensory neurons[1]. NGF released from the central processes in the spinal cord enhances the nociceptive response and contributes to central sensitization. The peripheral NGF release results in neurogenic inflammation[16]. Under inflammatory conditions, the retrograde transport of the produced NGF from the axon periphery to the cell body increases, and the neuropeptides and corresponding mRNA expressions increase as well[18,19]. Increased retrograde transport of NGF may change gene expression and thus lead to long lasting changes of neuron function[20]. In adult animals, the NGF produced in target tissues maintains the nociceptive properties of a subset of DRG neurons, which express a high affinity NGF receptor—tyrosine kinase receptor A (trkA). The NGF-responsive nociceptors consist of a subset of C fibers which express the neuropeptide SP and are sensitive to capsaicin[21]. Intrathecal injection of NGF has effects on C-fiber-evoked SP outflow and induces thermal hyperalgesia[22]. *In vivo*, deprivation of NGF by axotomy[23] or sequestration treatment with neutralizing anti-sera could reduce the sensitivity of DRG neurons to capsaicin and decrease the neuropeptide levels[24]. In cultured DRG neurons, NGF deprivation leads to a reversible loss and/or decrease of nociceptive properties, including capsaicin sensitivity[25], neuropeptides SP release and calcitonin gene-related peptide (CGRP) content[26]. Interestingly, NGF treatment only enhances the release of CGRP, but not SP, from the dorsal horn of spinal cord[27,28]. In the present study, the increment of SP mRNA in NGF-treated DRG was dependent on the dose of NGF. In previous study *in vitro*, the SP mRNA increment is consistent with the increasing mRNA levels of neuropeptides, such as CGRP, following the NGF treatment[16]. This result suggested that the SP synthesis may increase in cultured DRG neurons. SP mRNA at low expression may not be dependent upon NGF since SP mRNA is also expressed in the absence of exogenous NGF, but adequate exogenous NGF does stimulate higher expression of SP mRNA.

One property of the primary nociceptive afferent neurons regulated by NGF is sensitivity to capsaicin[25]. Capsaicin could depolarize DRG neurons[29] and evoke the release of the neuropeptides such as SP and CGRP[30]. In the present study, the SP basal release was just a little higher in NGF-treated neurons than in control group. The capsaicin-evoked SP release was four times than basal release in NGF-treated cultures. These results are coincidence with the previous studies *in vivo* and *in vitro* that NGF can increase the capsaicin responsiveness of neurons.

The expression pattern of VR1 may be associated to the capsaicin responsiveness of sensory neurons and pain sensation[31]. VR1 mRNA and protein could be detected at varying levels in small and medium sized nociceptive neurons including up to 90% of NGF responsive neurons[32]. VR1 mRNA could be down-regulated by axotomy, suggesting that peripherally derived trophic factors such as NGF may play an important role in VR1 expression under pathological conditions[33]. In the present study, the increment of VR1 mRNA was dependent on dose of NGF in primary cultured DRG neurons. This result suggests that the difference of responsiveness between the control culture and NGF-treated culture could be due to an increased responsiveness of NGF-treated DRG neurons.

Taken together, the results that NGF treatment increased both SP mRNA and VR1 mRNA expressions in cultured DRG neurons suggest that NGF regulates the increase of capsaicin-evoked SP partly through increasing the SP gene expression[34] and the VR1 receptor sensitivity[16]. Thus, upregulation of SP mRNA and VR1 mRNA may both contribute to the increment of capsaicin-evoked SP release.

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神经生长因子对培养的大鼠背根神经节神经元 P 物质释放的调节作用

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摘要: 目的 观察神经生长因子(nerve growth factor, NGF)对原代培养的背根神经节(dorsal root ganglion, DRG)神经元中P物质(substance P, SP)的基础释放量和辣椒素诱发释放量的调节效应。**方法** 将15 天胚龄的Wistar大鼠DRG神经元培养于含有不同浓度NGF的DMEM/F12培养液中，不含NGF的培养液培养的神经元作为对照。72 小时后，用RT-PCR检测神经元中SP mRNA和辣椒素受体(vanilloid receptor 1, VR1)mRNA的表达，用放射免疫分析(radioimmunoassay, RIA)法检测SP的基础释放量和辣椒素(100 nmol/L)刺激 10 min 后的诱发释放量。**结果** SP mRNA和VR1 mRNA在NGF孵育的标本中表达增加，并与孵育液中NGF的浓度呈剂量依赖关系。SP的基础释放量和辣椒素诱发释放量在NGF孵育的标本中均增加，而且诱发释放量与NGF的浓度呈剂量依赖关系。**结论** NGF使DRG神经元SP的基础释放量和诱发释放量增加，表明NGF能增加初级传入神经元感受伤害刺激的敏感性，该效应可能与SP和VR1的mRNA表达增加有关。

关键词: 神经生长因子；背根神经节；辣椒素；辣椒素受体；P 物质