Noradrenaline regulates substance P release from rat dorsal root ganglion neurons *in vitro*

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Abstract: Objective To determine whether activation and/or inhibition of α -adrenoreceptors influences substance P (SP) release from dorsal root ganglion (DRG) primary sensory neurons *in vitro*. **Methods** DRGs were dissected from 15-day embryonic Wistar rats. DRG neurons were dissociated and cultured for 2 d and then exposed to noradrenaline (NA) alone (1×10^{-4} mol/L), or along with the $\alpha 1$ -adrenoreceptor antagonist prazosin (1×10^{-6} mol/L) or the $\alpha 2$ -adrenoreceptor antagonist yohimbine (1×10^{-5} mol/L) for 4 d. Then, RT-PCR was used to determine the levels of preprotachykinin (PPT) mRNA encoding for SP and Western blot to assess the protein levels of SP. Basal and capsaicin (CAP)-evoked SP release were measured by enzyme-linked immunosorbent assay (ELISA). **Results** CAP-evoked SP release was sensitized by NA and this effect was inhibited by pre-incubation with prazosin but not with yohimbine. The levels of PPT mRNA, SP peptide, and basal SP release did not change significantly in any of the experimental conditions. **Conclusion** NA may significantly increase CAP-evoked SP release through activation of α -adrenoreceptors, which may contribute to noradrenergic pain modulation.

Keywords: noradrenaline; substance P; dorsal root ganglion

1 Introduction

The noradrenergic system is subject to plastic changes that influence its antinociceptive efficacy after injury or inflammation^[1,2]. Noradrenaline (NA) is a key neurotransmitter that activates adrenoreceptors under physiological and pathophysiological conditions. Interestingly, both $\alpha 1$ -^[3-5] and $\alpha 2$ -adrenoreceptors^[6-9] are expressed in dorsal root ganglion (DRG) primary sensory neurons and regulate neurogenic inflammation and nociceptive responses. NA depresses heat-activated K^+ channel currents by activating

$\alpha 1$ - but not $\alpha 2$ -adrenoreceptors in cultured rat DRG neurons^[10]. NA release also modulates nociception^[11,12]. Some of the DRG neurons freshly dissociated from rats with adjuvant-induced inflammation show a small depolarization in response to NA^[13].

Substance P (SP), an 11-amino acid neuropeptide found in sensory nerves innervating peripheral tissues^[14], is involved in inflammation and nociception. Primary sensory DRG neurons that respond to nociceptive stimuli are characterized by SP expression. SP release from the central processes activates second-order sensory neurons to transmit nociceptive signals into the central nervous system. Release of SP from peripheral endings causes a series of local inflammatory responses referred to as neurogenic inflammation^[5]. Interestingly, administration of vanilloids like resiniferatoxin or capsaicin (CAP) down-regulates SP

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in primary sensory neurons both *in vivo*^[15] and *in vitro*^[16]. SP release and SP levels in primary sensory neurons are influenced by many factors^[17-20]. A previous study has shown that the α 1-adrenoreceptor agonist phenylephrine causes the release of SP from terminals of CAP-sensitive sensory neurons in the lumbar enlargement of the dorsal spinal cord and urinary bladder^[5]. Neurogenic inflammation and pain resulting from CAP injection respond to NA released from sympathetic efferents^[21].

It remains unknown whether SP expression in, and SP release from DRG neurons are influenced by the activation or inhibition of α -adrenoreceptors. The present study aimed to address this question by exposing DRG neurons to the α -adrenoreceptor agonist NA, NA plus the α 1-adrenoreceptor antagonist prazosin, or the α 2-adrenoreceptor antagonist yohimbine *in vitro*, and assessing SP expression and release by RT-PCR, Western blot, and enzyme-linked immunosorbent assay (ELISA).

2 Materials and methods

2.1 Animals Embryonic 15-day Wistar rats were provided by the Experimental Animal Center of Shandong University, China.

2.2 DRG cell culture preparations Under aseptic conditions, the whole bilateral DRGs were removed and placed in Dulbecco's Modified Eagle's Medium with F-12 supplement (DMEM/F-12 medium) (Gibco; Grand Island, NY, USA). They were then digested with 0.25% trypsin (Sigma; St. Louis, MO, USA) in D-Hanks solution at 37 °C for 10 min, centrifuged, and triturated in culture medium. Dissociated DRG cells were then plated in poly-L-lysine-coated flasks (Costar; Corning, NY, USA) at a density of 5×10^5 cells/mL or in poly-L-lysine-coated 24-well clusters (Costar) at 1×10^5 cells/well. DRG cells were incubated at 37 °C with 5% CO₂ for 24 h and then maintained in culture medium containing cytarabine (5 µg/mL) for another 24 h to inhibit the growth of non-neuronal cells. Neurons were further cultured for another 4 d with medium being changed every 2 d. The composition of the culture medium was DMEM/F-12 (1:1) supplemented with 5% fetal bovine

serum(Gibco), 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). Cells cultured in flasks were used to detect the expression of preprotachykinin (PPT) mRNA encoding for SP by RT-PCR and SP peptide by Western blot analysis, while the cells in clusters were used to monitor SP levels using ELISA analysis.

2.3 Exposure of DRG neurons to NA DRG cells were allowed to grow processes for 2 d, and then incubated in culture medium containing NA (1×10^{-4} mol/L) for 4 d, with the medium being changed every 2 d. The DRG neurons were treated with the α 1-adrenoreceptor antagonist prazosin (1×10^{-6} mol/L) or the α 2-adrenoreceptor antagonist yohimbine (1×10^{-5} mol/L) 10 min prior to NA addition.

2.4 RNA extraction and RT-PCR After 4 d of drug treatment, cells were processed for RT-PCR. Total RNA was extracted from the cells in each flask using TRIzol (Invitrogen; Grand Island, NY, USA), and cDNA was synthesized with M-MLV reverse transcriptase. The primers for PPT and β -actin were as follows: PPT forward 5'-GCCCTTTGAGCATCTTCTTC-3', reverse 5'-GTCTGAGGAGGTCACCACAT-3'; β -actin forward 5'-ATCATGTTTGAGACCTTCAAC-3', reverse 5'-CATCTCTTGCTCGAAGTCCA-3'. β -actin served as the internal control. The predicted size of the amplified PPT DNA product was 450 bp and that of β -actin was 317 bp.

PCR was performed at 94 °C for 5 min, and 35 cycles at 94 °C for 45 s, 53 °C for 60 s, and 72 °C for 45 s, followed by the final 72 °C for 10 min. PCR was performed within the range which demonstrated a linear correlation between the amount of cDNA and the yield of PCR products.

The PCR products were analyzed by standard agarose gel electrophoresis, stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned and the electrophoresis gel images were analyzed using ImageJ 1.39u software. The level of PPT mRNA was expressed as the ratio to that of β -actin.

2.5 Western blot analysis of SP peptide expression DRG neurons were homogenized in 10 mmol/L Tris homog-

enization buffer (pH 7.4) containing protease inhibitors (Amersco; Solon, OH, USA), followed by centrifugation at 12 000 rpm for 20 min. The supernatant was collected and protein concentration was determined by using the BCA method (standard: BSA). Samples (50 µg) were resolved by 8% SDS gel and transferred to nitrocellulose membrane. After being blocked in blocking buffer (containing 5% nonfat milk) for 2 h at room temperature, the membranes were incubated with goat anti-SP polyclonal IgG (1:1 000; Santa Cruz Biotechnology; Santa Cruz, CA, USA) or mouse anti-β-actin monoclonal IgG (1:1 000; Santa Cruz Biotechnology) overnight at 4 °C. After washing (3×10 min), the membranes were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (1:2 000; Santa Cruz Biotechnology) or HRP-conjugated goat anti-mouse IgG (1:2 000; Santa Cruz Biotechnology). The membranes were washed again and proteins were visualized by an ECL Western blotting detection kit (Pierce Biotech; Rockford, IL, USA) on light-sensitive film. The films were scanned and the images were analyzed quantitatively using ImageJ. The level of SP was expressed as the ratio to that of β-actin.

2.6 ELISA analysis of SP release from DRG neurons

After 4 d of incubation with NA, prazosin plus NA, or yohimbine plus NA, DRG cell 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 incubated in release buffer for 10 min at 37 °C to measure basal SP release. Then, the buffer was replaced by fresh release buffer containing CAP (100 nmol/L) and cells were incubated for an additional 10 min to measure CAP-evoked SP release.

After each incubation, the incubation solutions were collected for measurement of SP release from DRG neurons using the SP ELISA kit (BACHEM; San Carlos, CA, USA) according to the manufacturer's instructions. Briefly, each well of the 96-well plate was loaded with 25 µL antiserum (except the blank wells), followed by incubation at room temperature for 1 h. Subsequently, 50 µL standard

SP dilutions or samples were added and incubated at room temperature for 2 h. Then 25 µL biotinylated tracer was added into each well (except the blank wells) and agitated gently, followed by incubation at 4 °C overnight. After washing 5 times with 300 µL/well EIA buffer, 100 µL HRP-conjugated streptavidin was added into each well. Then the plate was incubated at room temperature for 1 h and washed 5 times. Finally, 100 µL 3,3',5,5'-tetramethyl benzidine dihydrochloride was added into each well and the plate was incubated at room temperature for 30 min. The reaction was terminated by adding 100 µL 2 N HCl and absorbance was read at 450 nm using a microplate reader (Multiskan MK3, Thermo Labsystems, Finland) within 10 min.

2.7 Statistical analysis Data are expressed as mean ± SD and were analyzed with one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test using SPSS software (Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3 Results

3.1 PPT mRNA expression The ratio of PPT mRNA level to that of the control cells (cultured continuously in culture medium) in DRG cells treated with NA was 0.99 ± 0.11 , with NA plus prazosin was 1.20 ± 0.17 , and with NA plus yohimbine was 1.03 ± 0.19 . There values were not significant differences (Fig. 1).

3.2 SP peptide expression The relative density values for SP were: NA alone, (1.13 ± 0.18) -fold control; NA plus prazosin, (1.09 ± 0.16) -fold control; and NA plus yohimbine, (1.03 ± 0.11) -fold control. There were no significant differences between the groups (Fig. 2).

3.3 Basal and CAP-evoked SP release Administration of NA (1×10^{-4} mol/L) induced an increase in CAP-evoked SP release. Pretreatment with prazosin (1×10^{-6} mol/L) reduced the amount of CAP-evoked SP release compared to the NA alone, whereas yohimbine (1×10^{-5} mol/L) had no effect on the NA-induced increase of CAP-evoked SP release. In contrast, NA alone, prazosin plus NA, and yohimbine plus NA had no significant effect on basal SP release (Fig. 3).

Fig. 1 Effects of noradrenaline (NA) and α -adrenoreceptor antagonists on preprotachykinin (PPT) mRNA expression in primary cultured DRG neurons. **A:** RT-PCR results for PPT and β -actin. Lane 1, NA alone; lane 2, NA plus prazosin; lane 3, NA plus yohimbine; lane 4, control. **B:** Relative mRNA levels of PPT under different treatment conditions (mean $\pm$ SD; $n = 5$). There were no significant differences between the groups ($P > 0.05$).

4 Discussion

NA is a key neurotransmitter released by sympathetic postganglionic neurons and is critical in noradrenergic pain modulation^[1]. Both $\alpha 1$ - and $\alpha 2$ -adrenoreceptors have been identified in DRG neurons^[3-6,9]. NA activates adrenoreceptors to modulate noradrenergic pain. Both *in vivo* and *in vitro* studies have shown that the $\alpha 1$ -adrenoreceptor facilitates peripheral pain modulation^[22,23]. In contrast, accumulating evidence demonstrates that the $\alpha 2$ -adrenoreceptor is both facilitatory and inhibitory in peripheral pain modulation^[24,25]. However, whether $\alpha 2$ -adrenoreceptors in the periphery contribute to the aggravation or suppression of pain remains a matter of debate. In the present study, no significant difference in PPT mRNA (which encodes SP), SP peptide expression, or basal SP release was found in

Fig. 2 Effects of noradrenaline (NA) and α -adrenoreceptor antagonists on substance P (SP) expression in primary cultured DRG neurons. **A:** SP peptide analyzed by Western blot. Lane 1, NA alone; lane 2, NA plus prazosin; lane 3, NA plus yohimbine; lane 4, control. **B:** Quantitative analysis of SP protein levels. The levels of SP were expressed as the ratios to that of β -actin. SP relative expression in NA, NA plus prazosin, and NA plus yohimbine groups were normalized to that of the control group. There were no significant differences between the groups (mean $\pm$ SD; $n = 5$).

DRG neurons in response to NA and its blockers. However, NA increased CAP-evoked SP release, and this was inhibited by an $\alpha 1$ - but not by an $\alpha 2$ -adrenoreceptor antagonist. These results are consistent with a previous report that stimulation of cutaneous $\alpha 1$ -adrenoreceptors increases the excitability of heat-sensitized nociceptive afferents^[26].

SP localized in sensory neurons of the DRG is responsible for nociceptive and neuropathic pain transmission^[27,28]. Systemic or topical administration of vanilloids down-regulates SP in primary sensory neurons, suggesting that SP depletion was stimulated by CAP, which induces degeneration of a subpopulation of sensory afferents^[15,29]. Recent research has shown that sympathetic denervation significantly reduces the CAP-evoked expression of tran-

Fig. 3 Effects of noradrenaline (NA) and α -adrenoreceptor antagonists on substance P (SP) release from DRG neurons induced by capsaicin (CAP). NA alone, prazosin plus NA, and yohimbine plus NA had no effect on basal SP release ($P > 0.05$). Prazosin reduced the CAP-evoked SP release caused by NA, whereas yohimbine did not have this effect (mean $\pm$ SD; $n = 6$). * $P < 0.05$, ** $P < 0.01$.

sient receptor potential vanilloid type 1 and sensory neuropeptide calcitonin gene-related peptide^[21]. This suggests that sympathetic noradrenergic modulation is implicated in CAP-evoked pain sensitivity. Here we found that NA increased CAP-evoked but not basal SP release, implying that NA is not a tonic stimulator of SP release but increases the sensitivity to CAP.

The peripheral noradrenergic system is involved in the intrinsic control of pain. NA has little influence on pain in healthy tissues, whereas after injury or inflammation, the noradrenergic system is subject to plastic changes that influence its antinociceptive efficacy^[1]. Functional α -adrenoreceptors, which are expressed in primary sensory neurons, regulate neurogenic inflammation and nociceptive responses^[8]. α 1-adrenoreceptors mediate depolarization in cultured rat DRG neurons^[22]. α 1-adrenoreceptor agonists have been reported to increase and antagonists to reduce neurogenic inflammatory responses mediated by CAP-sensitive sensory neurons^[30,31]. The elevation of CAP-evoked SP release induced by NA found in the present study may correlate with noradrenergic pain modulation

by NA. This *in vitro* model provides new insights into the effects of activation or inhibition of α -adrenoreceptors expressed in primary sensory neurons on neurogenic inflammation and nociceptive responses.

In conclusion, NA, by acting on α 1- but not α 2-adrenoreceptors, increases CAP-evoked SP release from DRG neurons, indicating that it may be one of the pronociceptive noradrenergic mechanisms in the periphery.

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去甲肾上腺素对背根神经节神经元 P 物质释放的调节作用

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摘要: **目的** 观察激活或抑制 α -肾上腺素受体是否影响体外培养的背根神经节(dorsal root ganglion, DRG)神经元P物质(substance P, SP)的释放。**方法** 胎龄15天的Wistar大鼠DRG神经元培养2天后, 分别用去甲肾上腺素(noradrenaline, NA)(1×10^{-4} mol/L)、 α_1 -受体拮抗剂哌唑嗪(1×10^{-6} mol/L)+NA(1×10^{-4} mol/L)、 α_2 -受体拮抗剂育亨宾(1×10^{-5} mol/L)+NA(1×10^{-4} mol/L)孵育4天。用RT-PCR法检测DRG神经元编码SP蛋白的PPT mRNA表达水平, 用Western blot 法检测DRG神经元SP蛋白的表达水平, 用酶联免疫吸附测定法检测SP的基础释放量和辣椒素刺激后的释放量。**结果** NA单独孵育显著增加了DRG神经元辣椒素刺激后的SP释放量, α_1 -受体拮抗剂哌唑嗪预处理可阻断NA的效应, 而 α_2 -受体拮抗剂育亨宾不产生此作用。在各种实验条件下, PPT mRNA水平、SP蛋白表达水平和SP的基础释放量没有显著性差异。**结论** NA通过激活 α_1 -受体增加了DRG神经元辣椒素刺激后的SP释放量, 这一作用可能与去甲肾上腺素能的疼痛调节有关。

关键词: 去甲肾上腺素; P物质; 背根神经节