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## Effects of selective alpha 2-adrenoreceptor stimulation on capsaicin-evoked substance P release from primary cultured dorsal root ganglion neurons

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Alpha<sub>2</sub> adrenoreceptors are expressed in dorsal root ganglion (DRG) neurons and may be involved in inflammation or other physiological and pathophysiological conditions. To determine the effects of administration of selective alpha<sub>2</sub> adrenoreceptor agonists or antagonists on substance P (SP) release from DRG neurons without or with capsaicin stimulation, primary cultured embryonic rat DRG neurons were preincubated with the selective alpha<sub>2</sub> adrenoreceptor agonist clonidine (10<sup>-5</sup> mol/L) or the alpha<sub>2</sub> adrenoreceptor antagonist yohimbine (10<sup>-5</sup> mol/L) for 4 days, respectively, followed by the addition of capsaicin (10<sup>-7</sup> nmol/L) for additional 10 min. Cultures were examined by radioimmunoassay (RIA) for detecting SP levels released from DRG neurons before and after capsaicin stimulation. Expression of SP mRNA and vanilloid receptor 1 (VR1) mRNA was determined by RT-PCR. Administration of the selective alpha<sub>2</sub> adrenoreceptor agonist clonidine for 4 days could decrease capsaicin-evoked SP release but not basal SP release.

Administration of clonidine could decrease VR1 mRNA expression but not SP mRNA. Administration of the selective alpha<sub>2</sub> adrenoreceptor antagonist yohimbine did not have these effects. The inhibitory role of the selective alpha<sub>2</sub> adrenoreceptor agonist clonidine on capsaicin-evoked SP release may be through decreasing VR1 mRNA levels then reducing the sensitivity of nociceptors to capsaicin. The data provided in the present study suggest that adrenergic modulation on primary sensory neurons by an alpha<sub>2</sub> adrenoreceptor agonist may contribute to the analgesic effects in neurogenic inflammation.

### 1. Introduction

The sympathetic system (SNS) is considered to be a major component of the neurogenic contribution to inflammation and hyperalgesia (Safieh-Garabedian et al. 2002). The noradrenergic system is subject to various plastic changes that influence its antinociceptive efficacy after injury or inflammation (Pertovaara 2006). Sympathetic post-ganglionic neurons may be involved in the generation of pain, hyperalgesia and inflammation under pathophysiological conditions (Jänig et al. 1996). It has been demonstrated that alpha<sub>2</sub> adrenoreceptors are expressed in dorsal root ganglion (DRG) neurons (Gold et al. 1997; Shi et al. 2000; Ma et al. 2005). These adrenoreceptors are functionally active and may vary in the presence of nerve injury, inflammation or other physiological and pathophysiological conditions (Gold et al. 1997; Birder and Perl 1999; Ma et al. 2005).

Capsaicin, the pungent component of hot pepper, elicits a sensation of burning pain, via activation of vanilloid receptor 1 (VR1) expressed in primary sensory neurons (Winston et al.

2001; Tominaga et al. 2005). Capsaicin depolarizes DRG neurons (Oh et al. 1996; Lee et al. 2005) and evokes release of neuropeptide substance P (SP) (Hingtgen et al. 1995; Winston et al. 2001; Xing et al. 2006). SP, an 11-amino-acid peptide, is a member of the tachykinin family of peptide neurotransmitters that are derived from preprotachykinin gene by alternative splicing. SP is found in sensory nerves innervating peripheral tissues (Ribeiro-da-Silva and Hökfelt 2000). Release of SP from peripheral endings causes a series of local inflammatory responses referred to as neurogenic inflammation (Trevisani et al. 2007). Therefore, to provide the knowledge of the effects of administration of selective alpha<sub>2</sub> adrenoreceptor agonists or antagonists on capsaicin-evoked sensory neuropeptide release from dissociated cultured DRG neurons is important for understanding the adrenergic modulation on neurogenic inflammation and hyperalgesia. Here we investigated exposure of the alpha<sub>2</sub> adrenoreceptor agonist clonidine, or the alpha<sub>2</sub> adrenoreceptor antagonist yohimbine on SP release from dissociated cultured DRG neurons without or with capsaicin stimulation.

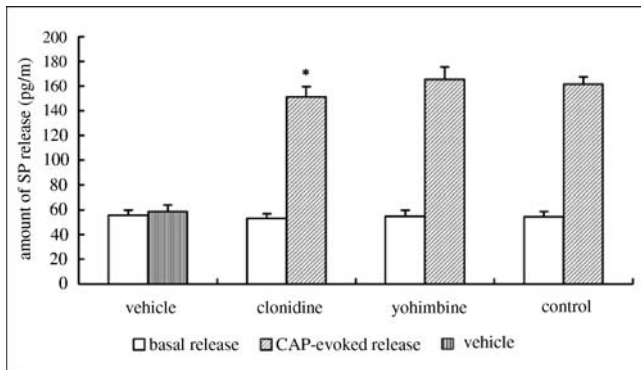


Fig. 1: Effects of clonidine or yohimbine on SP release from DRG neurons. Clonidine or yohimbine did not alter the basal SP release ( $P > 0.05$ ). Administration of the selective  $\alpha_2$  adrenoreceptor agonist clonidine for 4 days could decrease capsaicin-evoked SP release ( $P < 0.05$ ). The  $\alpha_2$  adrenoreceptor antagonist yohimbine did not have effect on capsaicin-evoked SP release ( $P > 0.05$ ). Bar graphs with error bars represent mean  $\pm$  SD ( $n = 6$ ). \* $P < 0.05$  vs. control

## 2. Investigations and results

### 2.1. Effects of exposure of clonidine or yohimbine on SP release from DRG neurons

Administration of the selective  $\alpha_2$  adrenoreceptor agonist clonidine ( $10^{-5}$  mol/L) for 4 days could decrease capsaicin-evoked SP release but not basal SP release.  $\alpha_2$  adrenoreceptor antagonist yohimbine ( $10^{-5}$  mol/L) did not have this effect (Fig. 1).

### 2.2. Effects of exposure of clonidine or yohimbine on SP mRNA expression of DRG neurons

Administration of the selective  $\alpha_2$  adrenoreceptor agonist clonidine ( $10^{-5}$  mol/L) or the  $\alpha_2$  adrenoreceptor antagonist yohimbine ( $10^{-5}$  mol/L) for 4 days did not have effect on SP mRNA expression (Fig. 2).

### 2.3. Effects of exposure of clonidine or yohimbine on VR1 mRNA expression of DRG neurons

Administration of the selective  $\alpha_2$  adrenoreceptor agonist clonidine ( $10^{-5}$  mol/L) for 4 days could decrease VR1 mRNA expression. The  $\alpha_2$  adrenoreceptor antagonist yohimbine ( $10^{-5}$  mol/L) did not affect VR1 mRNA expression (Fig. 3).

## 3. Discussion

The present study demonstrates that activation of  $\alpha_2$  adrenoreceptors by administration of the  $\alpha_2$  adrenoreceptor agonist clonidine could inhibit capsaicin-evoked sensory neuropeptide release and VR1 mRNA expression in primary cultured DRG neurons, inhibition of  $\alpha_2$  adrenoreceptors by administration of the  $\alpha_2$  adrenoreceptor antagonist yohimbine did not have effects on DRG neurons. These results implicated that the inhibitory role of an  $\alpha_2$  adrenoreceptor agonist is likely to be mediated by inhibiting VR1 expression to attenuate capsaicin sensitivity rather than by promoting neuropeptide synthesis. The present study provides important new evidence that adrenergic modulation by activation of  $\alpha_2$  adrenoreceptors to inhibit capsaicin-evoked neuropeptide release is under continuously stimulative states in primary DRG neuronal cultures.

However, it is still a matter of debate, which subtype of adrenoreceptor in the periphery contributes to aggravation of pain and which one to suppression of pain, since different adrenoreceptor

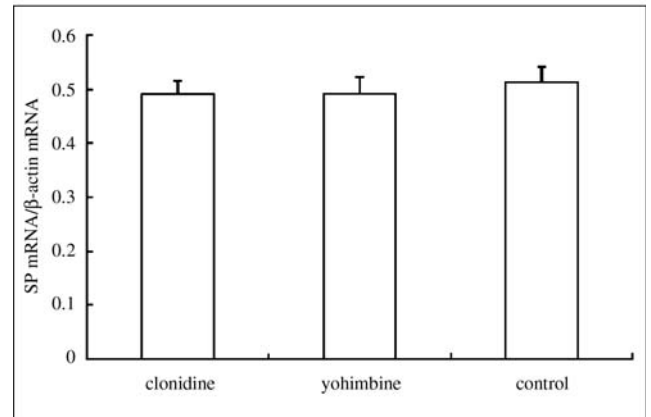
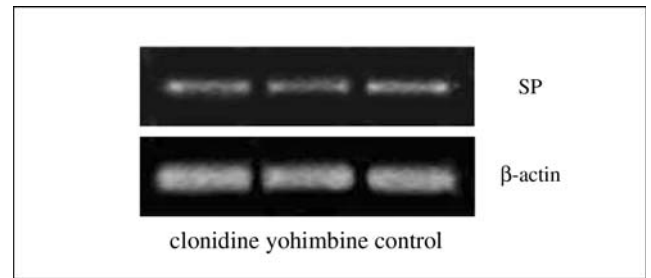


Fig. 2: Effects of clonidine or yohimbine on SP mRNA expression in primary cultured DRG neurons. SP and  $\beta$ -actin mRNA were analyzed by RT-PCR. The quantitative analysis of the results are as follows: DRG cells were treated with  $10^{-5}$  mol/L clonidine (SP mRNA/ $\beta$ -actin mRNA =  $0.4909 \pm 0.0248$ ). DRG cells were treated with  $10^{-5}$  mol/L yohimbine (SP mRNA/ $\beta$ -actin mRNA =  $0.4919 \pm 0.0315$ ). DRG cells were cultured continuously in growth media as control (SP mRNA/ $\beta$ -actin mRNA =  $0.5133 \pm 0.0279$ ). Administration of the selective  $\alpha_2$  adrenoreceptor agonist clonidine ( $10^{-5}$  mol/L) or the  $\alpha_2$  adrenoreceptor antagonist yohimbine ( $10^{-5}$  mol/L) for 4 days did not have effect on SP mRNA expression. Bar graphs with error bars represent mean  $\pm$  SD ( $n = 5$ )

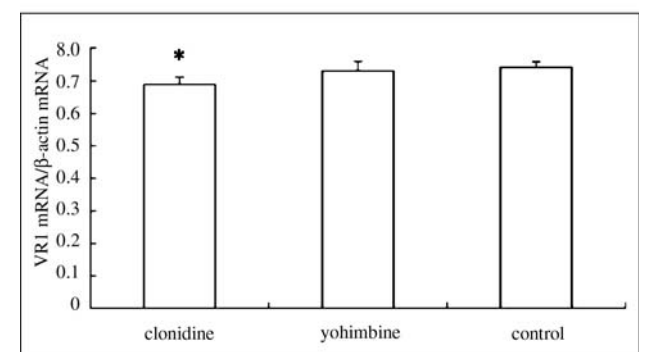
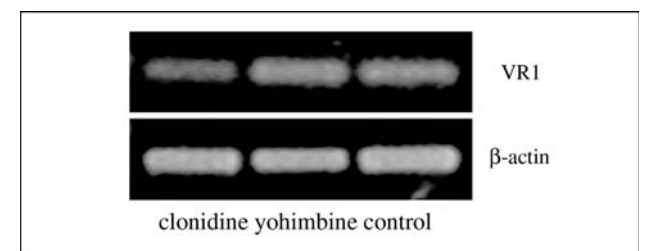


Fig. 3: Effects of clonidine or yohimbine on VR1 mRNA expression in primary cultured DRG neurons. VR1 and  $\beta$ -actin mRNA were analyzed by RT-PCR. The quantitative analysis of the results are as follows: DRG cells were treated with  $10^{-5}$  mol/L clonidine (VR1 mRNA/ $\beta$ -actin mRNA =  $0.6894 \pm 0.0232$ ). DRG cells were treated with  $10^{-5}$  mol/L yohimbine (VR1 mRNA/ $\beta$ -actin mRNA =  $0.7313 \pm 0.0296$ ). DRG cells were cultured continuously in growth media as control (VR1 mRNA/ $\beta$ -actin mRNA =  $0.7424 \pm 0.0173$ ). Bar graphs with error bars represent mean  $\pm$  SD ( $n = 5$ ). \* $P < 0.05$  vs. control

types have at least partly different functions and their functional effects varies with the pathophysiological condition (Pertovaara 2006). Both pain facilitatory role (Banik et al. 2001) and inhibitory role (Lavand'homme et al. 2002; Dogrul and Uzbay 2004) of peripheral  $\alpha_2$  adrenoreceptors were found in previous studies. Alpha 2-adrenoreceptor antagonist attenuated nerve injury-induced heat hyperalgesia suggested that alpha 2-adrenoreceptor may have a facilitatory role in mediating norepinephrine-induced pain sensation (Kingery et al. 2000; Banik et al. 2001). On the other hand, peripheral administration of an  $\alpha_2$  adrenoreceptor agonist attenuated nociceptive responses in control animals (Dogrul and Uzbay 2004) and hypersensitivity in inflammatory and neuropathic conditions (Lavand'homme et al. 2002; Wei et al. 2002) suggested that peripheral  $\alpha_2$  adrenoreceptors have a pain inhibitory role. Under continuously stimulative states of  $\alpha_2$  adrenoreceptor agonist clonidine, the inhibitory effect of capsaicin-evoked neuropeptide release may be relevant to the decreased VR1 expression. This result is consistent with the previous observations that  $\alpha_2$  adrenoreceptors were co-expressed with VR1 in DRG neurons and showed an inhibitory effect on hypersensitivity by administration of  $\alpha_2$  adrenoreceptor agonists at the sites of injured peripheral nociceptors and their DRG cell bodies (Ma et al. 2005). However, continuously exposure of the  $\alpha_2$  adrenoreceptor antagonist yohimbine has no effect on capsaicin-evoked neuropeptide release suggesting that the decreased activity of  $\alpha_2$  adrenoreceptors might be not enough to influence the sensitivity of DRG neurons to capsaicin at least the exposure time or concentration of the  $\alpha_2$  adrenoreceptor antagonist yohimbine used in the present study. Therefore, the inhibitory effects of  $\alpha_2$  adrenoreceptor agonists may play an important role in adrenergic pain modulation under pathophysiological conditions.

## 4. Experimental

### 4.1. DRG cell cultures

Dorsal root ganglia were dissected from embryonic 15-day-old Wistar rats obtained from the Experimental Animal Center of Shandong University of China. Dorsal root ganglia prior to establishment in culture were digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37 °C for 10 min, centrifuged, and triturated in growth media supplemented with 2.5% fetal bovine serum (Gibco). Dissociated DRG cells were then cultured in 24-well clusters (Costar, Corning, NY, USA) for monitoring SP levels using radioimmunoassay (RIA) or flasks (Costar, Corning, NY, USA) for detecting mRNAs for SP and VR1 by RT-PCR. The clusters and flasks were pre-coated with poly-L-lysine prior to plating DRG cells. DRG cells were plated at  $1 \times 10^5$  cells/well in clusters and at a density of  $5 \times 10^5$  cells/ml in flasks. Then DRG cells were cultured in culture media at 37 °C with 5% CO<sub>2</sub> for 24 h and then maintained in culture media containing cytarabine (ara-C) (5 µg/ml) for another 24 h to inhibit growth of non-neuronal cells, and then cultured in culture media for another 4 days with media change every 2 days.

### 4.2. Exposure of clonidine or yohimbine on DRG neurons and capsaicin treatment

DRG cell cultures were prepared as described above and allowed to grow processes for 2 days. Then the DRG neurons were incubated with the  $\alpha_2$  adrenoreceptor agonist clonidine ( $10^{-5}$  mol/L) or the  $\alpha_2$  adrenoreceptor antagonist yohimbine ( $10^{-5}$  mol/L) for 4 days with media change every 2 days. The culture media would contain clonidine ( $10^{-5}$  mol/L) or yohimbine ( $10^{-5}$  mol/L) during the 4 days incubation. Then the DRG neurons were examined by RT-PCR for SP mRNA and VR1 mRNA levels or stimulated by capsaicin ( $10^{-7}$  nmol/L) for 10 min before being examined by RIA. DRG neurons were cultured continuously in culture media for 6 days as control.

### 4.3. RT-PCR analysis for mRNAs for SP and VR1

The mRNA levels of SP and VR1 were analyzed by RT-PCR at 6 days of culture age with administration of the  $\alpha_2$  adrenoreceptor agonist clonidine or the  $\alpha_2$  adrenoreceptor antagonist yohimbine. The expression of  $\beta$ -actin was also determined as an internal control.

Total DRG cell RNA of each flask was isolated by TRIzol (Gibco). cDNA synthesis was performed with M-MLV reverse transcriptase. The gene-specific primers were synthesized by use of the published cDNA sequences for SP, VR1, and  $\beta$ -actin. The synthetic oligonucleotide primer sequences for SP, VR1, and  $\beta$ -actin were as follows: SP 5'-GCC CTT TGA GCA TCT TCT TC-3' (upper primer) and 5'-GTC TGA GGA GGT CAC CAC AT-3' (lower primer). VR1 5'-CTG ACG GCA AGG ATG ACT-3' (upper primer) and 5'-CCT AAG CAG ACC ACC CAA-3' (lower primer).  $\beta$ -Actin 5'-ATC ATG TTT GAG ACC TTC AAC-3' (upper primer) and 5'-CAT CTC TTG CTC GAA GTC CA-3' (lower primer). The predicted size of the amplified SP, VR1, and  $\beta$ -actin DNA products were 450 bp, 372 bp and 317 bp, respectively.

PCR amplification was performed for 35 cycles. The cycle profile included denaturation for 45 s at 94 °C, annealing for 60 s at 58 °C, and extension for 45 s at 72 °C. 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 and stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned and the electrophoresis gel images were analyzed quantitatively by using an ImageJ analysis software. The levels of SP and VR1 mRNA were expressed as the ratio of the gene to  $\beta$ -actin.

### 4.4. RIA analysis for SP release from DRG neurons

After 6 days of incubation at different treatment conditions, 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 incubated for 10 min at 37 °C in release buffer to measure basal SP release. Fresh release buffer containing capsaicin ( $10^{-7}$  mol/L) was added for an additional 10 min to measure capsaicin-evoked SP release. After each incubation, the culture media were removed and measured by RIA for SP release from DRG neurons.

The samples were reconstituted in PBS. Standards of synthetic SP (rat amino acid sequence) ranging from 2.5 to 1280 pg/assay tube dissolved in a volume of 0.2 ml PBS. The dissolved SP were then incubated at 4 °C with 0.1 ml of anti-SP antibody (anti-rat SP antibody) for 24 h. The mixture was then incubated for an additional 24 h at 4 °C with 0.1 ml of <sup>125</sup>I-labeled SP (20 000 counts/min/tube) in PBS. Free and bound neuropeptides were separated by adding 0.5 ml separating agent 45 min for SP. The RIA test tubes were centrifuged (4 000 rpm at 4 °C, 20 minutes). After removal of the supernatant fraction, the RIA test tubes were counted for iodine-125 remaining in the tubes.

### 4.5. Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical analysis was evaluated with SPSS software by one-way ANOVA followed by the Student-Newman-Keuls test for significance to compare the differences among various groups. Significance was accepted at  $P < 0.05$ .

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## References

- Banik RK, Sato J, Yajima H, Mizumura K (2001) Differences between the Lewis and Sprague-Dawley rats in chronic inflammation induced norepinephrine sensitivity of cutaneous C-fiber nociceptors. *Neurosci Lett* 299: 21–24.
- Birder LA, Perl ER (1999) Expression of  $\alpha_2$ -adrenergic receptors in rat primary afferent neurones after peripheral nerve injury or inflammation. *J Physiol* 515: 533–542.
- Dogrul A, Uzbay IT (2004) Topical clonidine antinociception. *Pain* 111: 385–391.
- Gold MS, Dastmalchi S, Levine JD (1997) Alpha 2-adrenergic receptor subtypes in rat dorsal root and superior cervical ganglion neurons. *Pain* 69: 179–190.
- Hingtgen CM, Waite KJ, Vasko MR (1995) Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3',5'-cyclic monophosphate transduction cascade. *J Neurosci* 15: 5411–5419.
- Jänig W, Levine JD, Michaelis M (1996) Interactions of sympathetic and primary afferent neurons following nerve injury and tissue trauma. *Prog Brain Res* 113: 161–184.
- Kingery WS, Guo TZ, Davies MF, Limbird L, Maze M (2000) The  $\alpha_2$  adrenoreceptor and the sympathetic postganglionic neuron contribute to the development of neuropathic heat hyperalgesia in mice. *Pain* 85: 345–358.

- Lavand'homme PM, Ma W, De Kock M, Eisenach JC (2002) Perineural alpha (2A)-adrenoceptor inhibits spinal cord neuroplasticity and tactile allodynia after nerve injury. *Anesthesiology* 97: 972–980.
- Lee Y, Lee CH, Oh U (2005) Painful channels in sensory neurons. *Mol Cells* 20: 315–324.
- Ma W, Zhang Y, Bantel C, Eisenach JC (2005) Medium and large injured dorsal root ganglion cells increase TRPV-1, accompanied by increased alpha2C-adrenoceptor co-expression and functional inhibition by clonidine. *Pain* 113: 386–394.
- Oh U, Hwang SW, Kim D (1996) Capsaicin activates a nonselective cation channel in cultured neonatal rat dorsal root ganglion neurons. *J Neurosci* 16: 1659–1667.
- Pertovaara A (2006) Noradrenergic pain modulation. *Prog Neurobiol* 80: 53–83.
- Ribeiro-da-Silva A, Hökfelt T (2000) Neuroanatomical localisation of substance P in the CNS and sensory neurons. *Neuropeptides* 34: 256–271.
- Safieh-Garabedian B, Poole S, Haddad JJ, Massaad CA, Jabbur SJ, Saadé NE (2002) The role of the sympathetic efferents in endotoxin-induced localized inflammatory hyperalgesia and cytokine upregulation. *Neuropharmacology* 42: 864–872.
- Shi TS, Winzer-Serhan U, Leslie F, Hökfelt T (2000) Distribution and regulation of alpha(2)-adrenoceptors in rat dorsal root ganglia. *Pain* 84: 319–330.
- Tominaga M, Tominaga T (2005) Structure and function of TRPV1. *Pflügers Arch* 451: 143–150.
- Trevisani M, Campi B, Gatti R, Andre E, Materazzi S, Nicoletti P, Gazzieri D, Geppetti P (2007) The influence of alpha(1)-adrenoreceptors on neuropeptide release from primary sensory neurons of the lower urinary tract. *Eur Urol* 52: 901–908.
- Wei H, Jyvasjarvi E, Niissalo S, Hukkanen M, Waris E, Kontinen YT, Pertovaara A (2002) The influence of chemical sympathectomy on pain responsivity and alpha 2-adrenergic antinociception in neuropathic animals. *Neuroscience* 114: 655–668.
- Winston J, Toma H, Shenoy M, Pasricha PJ (2001) Nerve growth factor regulates VR-1 mRNA levels in cultures of adult dorsal root ganglion neurons. *Pain* 89: 181–186.
- Xing Y, Liu Z, Wang LH, Huang F, Wang HJ, Li ZZ (2006) Butyrate sensitizes the release of substance P and calcitonin gene-related peptide evoked by capsaicin from primary cultured rat dorsal root ganglion neurons. *Neuroendocrinol Lett* 27: 695–701.