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SPECIAL REPORT

Anti-nociceptive responses produced by human putative counterpart of nocistatin

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b-nocistatin is a heptadecapeptide produced from bovine prepronociceptin and blocks the induction of hyperalgesia and touch-evoked pain (allodynia) by intrathecal administration of nociceptin or prostaglandin E_2 (PGE_2). Human prepronociceptin may generate a 30-amino acid peptide different in length from b-nocistatin. Here, we examine whether the human putative counterpart of nocistatin (h-nocistatin) possessed the same biological activities as b-nocistatin. Simultaneous intrathecal injection of h-nocistatin in mice blocked the induction of allodynia by nociceptin and PGE_2 in a dose-dependent manner with ID_{50} values of 329 pg kg^{-1} and 16.6 ng kg^{-1}, respectively. h-nocistatin was about 10 times less potent than b-nocistatin. h-nocistatin also attenuated the nociceptin- and PGE_2-induced hyperalgesia. These results demonstrate that h-nocistatin is biologically active and may be involved in the processing of pain at the spinal level in humans.

Keywords: Nocistatin; nociceptin/orphanin FQ; prostaglandin E_2; allodynia; hyperalgesia; spinal cord

Introduction In relation to clinically relevant hyperalgesic states such as inflammation and neuropathic pain, there is considerable interest in neurochemical mechanisms of hyperalgesia and touch-evoked pain (allodynia) (Dray *et al*., 1994; Woolf, 1994). The dorsal horn of the spinal cord is an important site for pain transmission and many substances are involved in the modulation of incoming pain information (Yaksh & Aimone, 1989). We demonstrated for the first time that intrathecal (*i.t.*) administration of prostaglandin (PG) E_2 and $PGF_{2\alpha}$ induced allodynia in conscious mice among naturally occurring substances. Mechanisms of allodynia and hyperalgesia induced by PGs have been extensively characterized (Minami *et al*., 1992; 1994a, b, 1996; 1997b).

The heptadecapeptide called nociceptin/orphanin FQ (hereafter nociceptin) has been identified as an endogenous ligand of the orphan opioid-like receptor ORL_1 or ROR-C (Meunier *et al*., 1995; Reinscheid *et al*., 1995; Okuda-Ashitaka *et al*., 1996) and suggested to be involved in the regulation of nociceptive processing in the spinal cord (Reinscheid *et al*., 1995; Xu *et al*., 1996; Tian *et al*., 1997). We have shown that *i.t.* administration of nociceptin induces allodynia as well as hyperalgesia (Okuda-Ashitaka *et al*., 1996; Hara *et al*., 1997). Recently, we have revealed that bovine prepronociceptin contains another biologically active heptadecapeptide named nocistatin (Okuda-Ashitaka *et al*., 1998). b-nocistatin blocks allodynia and hyperalgesia induced by nociceptin and PGE_2. In contrast to nociceptin, amino acid sequences of prepronociceptin corresponding to b-nocistatin are not well conserved among species. While bovine prepronociceptin contains pairs of basic amino acids Lys-Arg, a general cleavage site for precursor maturation, at positions 109–110 and 128–129, the human and mouse precursors are devoid of the former cleavage site, which may generate 30- and 41-amino acid peptides, respectively, larger than the heptadecapeptide b-nocistatin (Okuda-Ashitaka *et al*., 1998). In the present study, we examined the effects of the human putative counterpart of nocistatin (h-nocistatin) on pain responses *in vivo*.

Methods Male ddY mice weighing 20 ± 2 g were used. Agents in vehicle (5 μl) were injected slowly into the subarachnoid space between the L_5 and L_6 vertebrae of conscious mice by a slight modification of the method of Hylden & Wilcox (1980). Control mice were given saline. Allodynia was assessed by light stroking of the flank of the mice with a paintbrush as described previously (Okuda-Ashitaka *et al*., 1998). For hyperalgesia, mice were placed on a hot plate maintained at 55°C, and the elapsed time until the mice showed the first avoidance responses (licking the feet, jumping or rapidly stamping the paws) was measured 15 or 30 min after *i.t.* injection of nociceptin and PGE_2, respectively. h-nocistatin (MPRVRSLFQEQEEPEPGMEEAGEMEQKQLQ) used in this study was synthesized by the solid-phase method and purified by high-performance liquid chromatography. All agents, including saline, were coded to assure blind testing. The animals were used only for one measurement in each experiment. This study was conducted with the approval of the local ethics committee and in accordance with the guidelines of the Ethics Committee of the International Association for the Study of Pain (Zimmermann, 1983). Data for allodynia were analysed by non-parametric ANOVA and statistical significance ($P<0.05$) was further examined by Williams' test for multiple comparison. ID_{50} values with 95% confidence limits (95% CL) were calculated using the computer program of Probit test. Data for hyperalgesia were analysed by parametric ANOVA and statistical significance ($P<0.05$) was further examined by Duncan's test.

Results When tactile stimuli were applied to the flank every 5 min after (*i.t.*) injection of 2.5 ng kg^{-1} nociceptin, prominent

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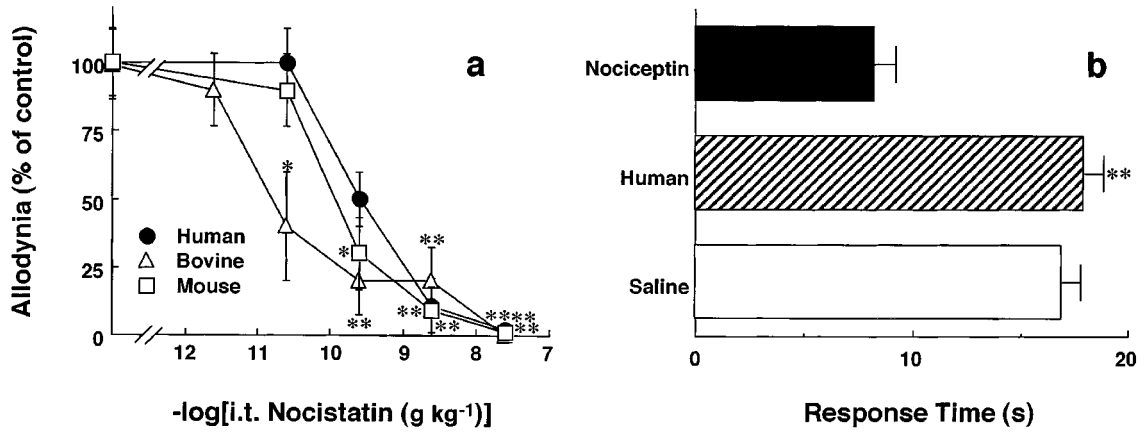

Figure 1 Effects of h-nocistatin on nociceptin-induced pain responses. (a) Allodynia. Nociceptin (2.5 ng kg^{-1}) was injected simultaneously with the indicated doses of b-nocistatin, h-nocistatin or m-nocistatin into the subarachnoid space of conscious mice. Allodynia was assessed at 10 min after *i.t.* injection as described previously (Okuda-Ashitaka *et al*., 1998). The allodynic score at 10 min after *i.t.* injection of nociceptin alone was 83.3% of the maximum possible score and was taken as 100%. The values shown are the mean ± s.e.mean ($n=6$). Statistical analyses were carried out by Willaims' test. $^{*}P<0.05$, $^{**}P<0.01$, as compared with the nociceptin-injected group. (b) Hyperalgesia. Nociceptin (2.5 ng kg^{-1}) was injected simultaneously with or without 25 ng kg^{-1} of h-nocistatin into the subarachnoid space. Hyperalgesia was assessed at 15 min after *i.t.* injection of nociceptin. The values shown are the mean ± s.e.mean ($n=10$). Statistical analyses were carried out by Duncan's test. $^{**}P<0.01$, as compared with the nociceptin-injected group.

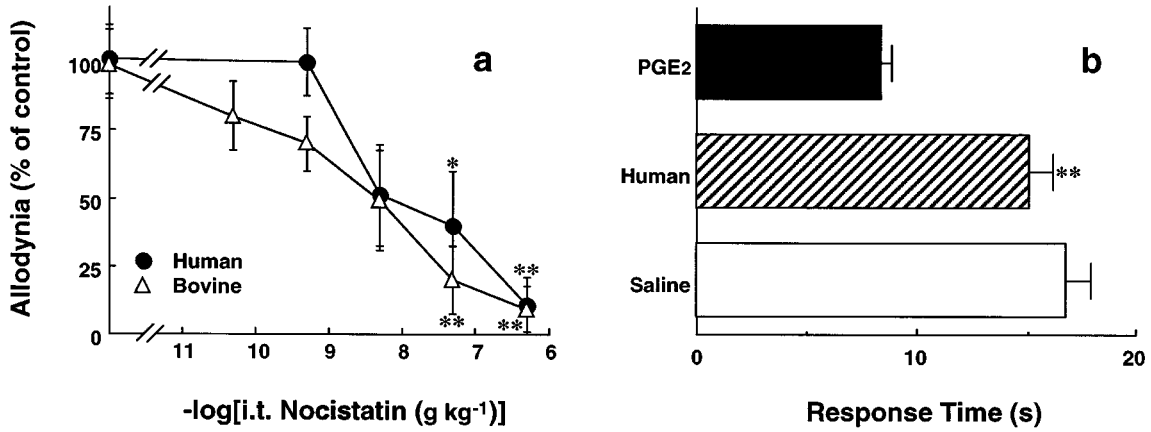

Figure 2 Effects of h-nocistatin on PGE_2-induced pain responses. (a) Allodynia. PGE_2 (500 ng kg^{-1}) was injected simultaneously with the indicated doses of b-nocistatin or h-nocistatin into the subarachnoid space of conscious mice. Allodynia was assessed at 5 min after *i.t.* injection of PGE_2 as described in the legend for Figure 1. The allodynic score of PGE_2 alone was 83.3% of the maximum possible score and was taken as 100%. (b) Hyperalgesia. PGE_2 (500 ng kg^{-1}) was injected simultaneously with or without 500 ng kg^{-1} of h-nocistatin into the subarachnoid space. Hyperalgesia was assessed at 30 min after *i.t.* injection of PGE_2.

allodynic responses such as vocalization, biting, and escape from the probe, were observed in all mice treated with nociceptin at 5 min after *i.t.* injection and continued over the 50 min experimental period. The effect of h-nocistatin on the nociceptin-induced allodynia was evaluated at 10 min, the point of the maximum allodynic effect achieved by 2.5 ng kg^{-1} nociceptin. Simultaneous injection of h-nocistatin blocked the nociceptin-induced allodynia in a dose-dependent manner with an ID_{50} value (95% CL) of 329 pg kg^{-1} (87.1 pg kg^{-1}–1.16 ng kg^{-1}) (Figure 1a). This value is comparable to that (162 pg kg^{-1} (22.5–661 pg kg^{-1})) of a mouse putative counterpart of nocistatin (m-nocistatin), but one order higher than that (35.8 pg kg^{-1} (1.70–213 pg kg^{-1})) of b-nocistatin. The *i.t.* administration of b-nocistatin, h-nocistatin, m-nocistatin at doses examined or saline did not induce allodynia by itself.

The hyperalgesic effect was addressed by the hot plate (55°C) test. There was no significant difference in the latency period of withdrawal between mice without *i.t.* injection (15.8 ± 0.8 s, mean ± s.e.mean, $n=10$) and mice treated with *i.t.* saline (16.9 ± 0.9 s, at 15 min). Compared with saline (Figure 1b), nociceptin (2.5 ng kg^{-1}) shortened the latency period of foot withdrawal to 8.2 ± 1.0 s at 15 min after *i.t.* injection. The nociceptin-evoked hyperalgesia was completely reversed by co-injection of h-nocistatin (25 ng kg^{-1}) to the control level (17.9 ± 1.0 s).

Intrathecal administration of PGE_2 also induced allodynia and hyperalgesia in conscious mice (Minami *et al*., 1994a). The effect of h-nocistatin on PGE_2-induced allodynia was evaluated at 5 min after *i.t.* administration of 500 ng kg^{-1} PGE_2. h-nocistatin blocked the PGE_2-induced allodynia in a dose-dependent manner with an ID_{50} of 16.6 ng kg^{-1} (2.48–

112 ng kg^{-1}), one order higher than that (2.84 ng kg^{-1}) for b-nocistatin (Figure 2a). As shown in Figure 2b, h-nocistatin (500 ng kg^{-1}) also reversed the PGE_2 (500 ng kg^{-1})-induced hyperalgesia (8.4 ± 0.5 s) to 15.1 ± 1.1 s at 30 min after *i.t.* injection, comparable to the control level (16.8 ± 1.1 s, at 30 min).

Discussion We have recently showed that the carboxyl-terminal hexapeptide of nocistatin (EQKQLQ), which is conserved in bovine, human, and mouse species, appears to be a minimal essential core for biological activity (Okuda-Ashitaka *et al*., 1998). Consistent with this notion, here we demonstrate that the allodynia evoked by nociceptin was dose-dependently blocked by h- and m-nocistatin. h-nocistatin also reversed the nociceptin-evoked hyperalgesia and the PGE_2-induced pain responses, demonstrating that h-nocistatin exhibited similar biological activity to b-nocistatin. The ID_{50} values of h-nocistatin for nociceptin- and PGE_2-induced allodynia are approximately one order of magnitude higher than those of b-nocistatin. This may be due to difference in species or in the length of the peptides. We previously showed that PGD_2 attenuated both nociceptin- and PGE_2-induced allodynia (Minami *et al*., 1996; 1997a). Nocistatin and PGD_2 may modulate a site of pain transmission common to nociceptin and PGE_2. However, PGD_2 did not affect the nociceptin- and PGE_2-induced hyperalgesia. This confirmation of the biological activity of h-nocistatin may accelerate the elucidation of neurochemical mechanism of action of nocistatin in spinal pain transmission.

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References

DRAY, A., URBAN, L. & DICKENSON, A. (1994). Pharmacology of chronic pain. *Trends Pharmacol. Sci.,* **15,** 190–197.

HARA, N., MINAMI, T., OKUDA-ASHITAKA, E., SUGIMOTO, T., SAKAI, M., ONAKA, M., MORI, H., IMANISHI, T., SINGU, K. & ITO, S. (1997). Characterization of nociceptin hyperalgesia and allodynia in conscious mice. *Br. J. Pharmacol.,* **121,** 401–408.

HYLDEN, J.L.K. & WILCOX. G.L. (1980). Intrathecal morphine in mice: a new technique. *Eur. J. Pharmacol.,* **67,** 313–316.

MEUNIER, J.-C., MOLLEREAU, C., TOLL, L., SUAUDEAU, C., MOISAND, C., ALVINERIE, P., BUTOUR, J.-L., GUILLEMOT, J.-C., FERRARA, P., MONSARRAT, B., MAZARGUIL, H., VASSART, G., PARMENTIER, M. & COSTENTIN, J. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL_1 receptor. *Nature,* **377,** 532–535.

MINAMI, T., NISHIHARA, I., UDA, R., ITO, S., HYODO, M. & HAYAISHI, O. (1994a). Characterization of EP-receptor subtypes involved in allodynia and hyperalgesia induced by intrathecal administration of prostaglandin E_2 to mice. *Br. J. Pharmacol.,* **112,** 735–740.

MINAMI, T., NISHIHARA, I., UDA, R., ITO, S., HYODO, M. & HAYAISHI, O. (1994b). Involvement of glutamate receptors in allodynia induced by prostaglandins E_2 and $F_{2\alpha}$ injected to conscious mice. *Pain,* **57,** 225–231.

MINAMI, T., OKUDA-ASHITAKA, E., MORI, H., ITO, S. & HAYAISHI, O. (1996). Prostaglandin D_2 inhibits prostaglandin E_2-induced allodynia in conscious mice. *J. Pharmacol. Exp. Ther.,* **278,** 1146–1152.

MINAMI, T., OKUDA-ASHITAKA, E., NISHIZAWA, M., MORI, H. & ITO, S. (1997a). Inhibition of nociceptin-induced allodynia in conscious mice by prostaglandin D_2. *Br. J. Pharmacol.,* **122,** 605–610.

MINAMI, T., SUGATANI, J., SAKIMURA, K., ABE, M., MISHINA, M. & ITO, S. (1997b). Absence of prostaglandin E_2-induced hyperalgesia in NMDA receptor ε subunit knockout mice. *Br. J. Pharmacol.,* **120,** 1522–1526.

MINAMI, T., UDA, R., HORIGUCHI, S., ITO, S., HYODO, M. & HAYAISHI, O. (1992). Allodynia evoked by intrathecal administration of prostaglandin $F_{2\alpha}$ to conscious mice. *Pain,* **50,** 223–229.

OKUDA-ASHITAKA, E., MINAMI, T., TACHIBANA, S., YOSHIHARA, Y., NISHIUCHI, Y., KIMURA, T. & ITO, S. (1998). Nocistatin, a peptide that blocks nociceptin action in pain transmission. *Nature,* **392,** 286–289.

OKUDA-ASHITAKA, E., TACHIBANA, S., HOUTANI, T., MINAMI, T., MASU, Y., NISHI, M., TAKESHIMA, H., SUGIMOTO, T. & ITO, S. (1996). Identification and characterization of an endogenous ligand for opioid receptor homologue ROR-C: its involvement in allodynic response to innocuous stimulus. *Mol. Brain Res.,* **43,** 96–104.

REINSCHEID, R.K., NOTHACKER, H.-P., BOURSON, A., ARDATI, A., HENNINGSEN, R.A., BUNZOW, J.R., GRANDY, D.K., LANGEN, H., MONSMA, F.J. JR & CIVELLI, O. (1995). Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. *Science,* **270,** 792–794.

TIAN, J.-H., XU, W., FANG, Y., MOGIL, J.S., GRISEL, J.E., GRANDY, D.K. & HAN, J.-S. (1997). Bidirectional modulatory effect of orphanin FQ on morphine-induced analgesia: antagonism in brain and potentiation in spinal cord of the rat. *Br. J. Pharmacol.,* **120,** 676–680.

WOOLF, C.J. (1994). The dorsal horn: state-dependent sensory processing and the generation of pain. In: *Textbook of Pain*, 3rd Edn, eds. Wall, P.D. & Melzack, R. pp. 101–112. Edinburgh: Churchill Livingstone.

XU, X.-J., HAO, J.-X. & WIESENFELD-HALLIN, Z. (1996). Nociceptin or antinociceptin: potent spinal antinociceptive effect of orphanin FQ/nociceptin in the rat. *NeuroReport,* **7,** 2092–2094.

YAKSH, T.L. & AIMONE, L. (1989). The central pharmacology of pain transmission. In: *Textbook of Pain*, 2nd Edn, eds. Wall, P.D. & Melzack, R. pp. 283–320. Edinburgh: Churchill Livingstone.

ZIMMERMANN, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain,* **16,** 109–110.

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