Note

Angiotensin (1–7) Attenuates the Nociceptive Behavior Induced by Substance P and NMDA *via* Spinal MAS1

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The intrathecal (i.t.) injection of substance P (SP) and *N*-methyl-D-aspartate (NMDA) induce transient nociceptive response by activating neurokinin (NK) 1 and NMDA receptors, respectively. We have recently reported that angiotensin (Ang) (1–7), an N-terminal fragment of Ang II, could alleviate several types of pain including neuropathic and inflammatory pain by activating spinal MAS1. Here, we investigated whether Ang (1–7) can inhibit the SP- and NMDA-induced nociceptive response. The nociceptive response induced by an i.t. injection of SP or NMDA was assessed by measuring the duration of hindlimb scratching directed toward the flank, biting and/or licking of the hindpaw or the tail for 5 min. Localization of MAS1 and either NK1 or NMDA receptors in the lumbar superficial dorsal horn was determined by immunohistochemical observation. The nociceptive response induced by SP and NMDA was attenuated by the i.t. co-administration of Ang (1–7) (0.03–3 pmol) in a dose-dependent manner. The inhibitory effects of Ang (1–7) (3 pmol) were attenuated by A779 (100 pmol), a MAS1 antagonist. Moreover, immunohistochemical analysis showed that spinal MAS1 co-localized with NK1 receptors and NMDA receptors on cells in the dorsal horn. Taken together, the i.t. injection of Ang (1–7) attenuated the nociceptive response induced by SP and NMDA *via* spinal MAS1, which co-localized with NK1 and NMDA receptors. Thus, the spinal Ang (1–7)/MAS1 pathway could represent a therapeutic target to effectively attenuate spinal pain transmission caused by the activation of NK1 or NMDA receptors.

Key words angiotensin (1–7); MAS1; substance P; *N*-methyl-D-aspartate; spinal cord

INTRODUCTION

Neurokinin (NK) 1 and *N*-methyl-D-aspartate (NMDA) receptors are known to play a role in the facilitation of spinal pain transmission. In the spinal cord, NK1-positive projections mediate spinal nerve ligation (SNL)-induced neuropathic pain¹⁾ as well as facet joint injury induced nociception.²⁾ In addition, the activation of spinal NMDA receptors was shown to be involved in chemotherapy⁻³⁾ and SNL-induced neuropathic pain.⁴⁾ Substance P (SP) and NMDA act as full agonists at NK1 receptors and NMDA receptors, respectively, and the intrathecal (i.t.) injection of these agonists has been shown to induce nociceptive behavior.^{5,6)}

Angiotensin (Ang) (1–7), an N-terminal fragment of Ang II, is mainly formed from Ang II by the action of Ang-converting enzyme (ACE) 2. By acting on the G protein-coupled receptor MAS1, Ang (1–7) exerts a counter-regulatory effect towards the Ang II/AT1 receptor axis. Hence, the ACE2/Ang (1–7)/MAS1 axis is considered as an inhibitory axis of the renin-Ang system. The i.t. injection of Ang II was shown to produce a nociceptive behavior *via* spinal AT1 receptors⁷⁾ that could be abolished by the i.t. injection of Ang (1–7) acting through MAS1.⁸⁾ The i.t. injection of Ang (1–7) also alleviated the hyperalgesia observed in type 1⁹⁾ and type 2¹⁰⁾ diabetic mice as well as formalin-induced inflammatory pain.¹¹⁾ Together, these findings suggest that Ang (1–7) plays an inhibitory role in spinal pain transmission. However, the effect of Ang (1–7) on the nociceptive response induced by the i.t. injection of SP and

NMDA remains unknown.

Thus, in the present study, we investigated the effects of Ang (1–7) on nociceptive response induced by the i.t. injection of SP and NMDA. Moreover, we examined the possible colocalization of MAS1 with either NK1 receptors or NMDA receptors in the lumbar superficial dorsal horn.

MATERIALS AND METHODS

Animals Male ddY mice (22–24 g; Japan SLC, Japan) were used in this study. These were housed in cages with free access to food and water under controlled conditions (temperature, 22 ± 2 °C; humidity, 55 ± 5%) and a 12/12 h light–dark cycle (lights on: 07:00 to 19:00). Groups of 10 mice for behavioral experiments and 3 mice for immunohistochemical experiments were used in single experiments. All experiments were performed following the approval from the Ethics Committee of Animal Experiment at Tohoku Medical and Pharmaceutical University (Approval No. A14023-cn).

Drugs and Antibodies The following drugs and reagents were used: Ang (1–7) and SP (Peptide Institute, Japan); NMDA (Nacalai Tesque, Japan); [D-Ala⁷]-Ang (1–7) (A779) (Bachem, Switzerland); rabbit anti-MAS1 antibody (#AAR-013), rabbit anti-NK1 receptor antibody (#ATR-001) and rabbit anti-NMDA receptor 1 (GluN1 subunit) antibody (#AGC-001; Alomone Laboratories, Israel); Alexa Fluor 488-conjugated AffiniPure Fab fragment goat anti-rabbit immunoglobulin G (IgG) (#111-547-003; Jackson ImmunoResearch Laboratories, U.S.A.) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (#A-11011; Thermo Fisher Scientific, U.S.A.); 4',6-diamidino-

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Fig. 1. Effect of Ang (1–7) on SP-Induced Nociceptive Behavior in Mice

Vehicle, Ang (1–7) (0.03–3 pmol), or Ang (1–7) (3 pmol) in combination with A779 (30 or 100 pmol) were co-administered i.t. with SP (0.1 nmol). A779 (100 pmol) or vehicle alone were injected as controls. The duration of scratching, biting and licking induced by SP was determined over a 5 min period starting immediately after its administration. One-way ANOVA: $F_{7,72} = 29.61$, $p < 0.01$. Values represent the means $\pm$ standard error of the mean (S.E.M.) for 10 mice per group. ** $p < 0.01$ compared with vehicle-injected controls, ## $p < 0.01$ compared with SP alone and †† $p < 0.01$ compared with Ang (1–7) (3 pmol) in combination with SP.

2-phenylindole dihydrochloride (DAPI) (Dojindo, Japan); normal goat serum (NGS) (Invitrogen, U.S.A.).

Intrathecal Injections The i.t. injections were performed in unanesthetized mice at the L5, L6 intervertebral space as previously described.⁷⁾ Briefly, a volume of 5 μ L was administered i.t. with a 28-gauge needle connected to a 50- μ L Hamilton microsyringe, the animal being lightly restrained to ensure needle positioning. Puncture of the dura was indicated behaviorally by a slight flick of the tail. For i.t. injections, all drugs were dissolved in Ringer's solution.

Measurement of Nociceptive Behavior At least 30 min before the i.t. injection, the mice were habituated to the transparent cage (22.0 $\times$ 15.0 $\times$ 12.5 cm). Immediately after the injection, the mice were placed individually in this cage and the accumulated response time of hindlimb scratching directed toward the flank, biting and/or licking of the hindpaw or the tail was measured during a 5 min period.

Immunohistochemical Staining The immunohistochemical labeling for MAS1 was performed in conjunction with NK1 receptors or NMDA receptor GluN1 subunit. Since the primary antibodies used to detect these three receptors were all derived from same host species (rabbit), sequential labeling was performed using Fab fragments, instead of whole antibodies, for the first target (MAS1). Spinal cord slices were stained as previously described.¹⁰⁾ Briefly, after blocking with phosphate-buffered saline (PBS) containing 10% NGS, the slices were incubated with anti-Mas1 antibody (1:100, 4°C, overnight). The slices were washed several times with PBS and incubated with Alexa Fluor 488-conjugated Fab fragment IgG (1:80, 4°C, overnight). After rinsing with PBS, the slices were incubated with anti-NK1 or -GluN1 antibody (1:100,

Fig. 2. Effect of Ang (1–7) on NMDA-Induced Nociceptive Behavior in Mice

Vehicle, Ang (1–7) (0.03–3 pmol), or Ang (1–7) (3 pmol) in combination with A779 (30 or 100 pmol) were co-administered i.t. with NMDA (0.3 nmol). Vehicle alone was used as reference. The duration of scratching, biting and licking induced by NMDA was determined over a 5 min period starting immediately after its administration. One-way ANOVA: $F_{6,63} = 12.67$, $p < 0.01$. Values represent the means $\pm$ S.E.M. for 10 mice per group. ** $p < 0.01$ compared with vehicle-injected controls, ## $p < 0.01$ compared with NMDA (0.3 nmol) alone and †† $p < 0.01$ compared with Ang (1–7) (3 pmol) in combination with NMDA (0.3 nmol).

4°C, overnight) followed by an incubation with Alexa Fluor 568-conjugated IgG (1:200, 4°C, overnight). All antibodies were diluted in PBS containing 1% NGS. The observation of immunofluorescent signals and subsequent cell counting were conducted using a Nikon C2 confocal microscope system (Nikon, Japan).

Statistical Methods Data were expressed as means $\pm$ standard error of the mean (S.E.M.). Significant differences were analyzed by a one-way ANOVA, followed by Tukey's multiple comparison test. All statistical analyses were performed using GraphPad Prism software, version 7.03 (GraphPad Software, U.S.A.). In all comparisons, $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Ang (1–7) on SP-Induced Nociceptive Behavior The i.t. injection of SP induces a nociceptive response consisting of scratching, biting and licking in mice.⁵⁾ To examine whether Ang (1–7) can inhibit the nociceptive response induced by SP (0.1 nmol), varying doses of Ang (1–7) (0.03–3 pmol) were co-administered i.t. As illustrated in Fig. 1, we found that Ang (1–7) suppressed the nociceptive response induced by SP in a dose-dependent manner. We have previously determined that a 30–100 fold higher dose of the MAS1 antagonist A779 was capable of antagonizing the inhibition of Ang (1–7) on diabetic neuropathic pain^{9,10)} as well as on the second phase of the formalin-induced nociception.¹¹⁾ Accordingly, the i.t. administration of A779 (100 pmol), a MAS1 antagonist, completely prevented the anti-nociceptive effect of Ang (1–7) (3 pmol), while A779 (100 pmol) alone did not affect

the basal behavioral response (Fig. 1). These results suggest that Ang (1–7) attenuates the SP-induced nociceptive behavior *via* spinal MAS1.

Effect of Ang (1–7) on NMDA-Induced Nociceptive Behavior NMDA is also known to induce a nociceptive behavior when it is administered i.t.⁶⁾ Therefore, we examined the potential counter-effect of Ang (1–7) on the NMDA-induced nociceptive behavior. Indeed, the NMDA-induced behavior (0.3 nmol) was again dose-dependently suppressed by the i.t. co-administration of Ang (1–7) (0.03–3 pmol), and this inhibition was significantly reversed by the i.t. injection of A779 (100 pmol) (Fig. 2). These results suggest that Ang (1–7) attenuates the NMDA-induced nociceptive behavior *via* spinal

MAS1.

Co-localization of MAS1 and NK1 Receptors or GluN1 Subunit in the Lumbar Superficial Dorsal Horn Immunohistochemical experiments were performed to determine whether spinal NK1 receptors or GluN1 subunit co-localized with MAS1. We found that both NK1 receptors (Fig. 3a) and GluN1 subunit (Fig. 3b) were expressed in MAS1-immunoreactive cells. Since we used primary antibodies from the same host species in each dual labeling, high concentrations of Alexa 488-conjugated Fab fragments were used as secondary antibody against the anti-MAS1 antibody. The rationale for this was to saturate and block the antigen binding sites on the primary rabbit anti-MAS1 antibody with the label thereby

Fig. 3. Double Immunohistochemical Staining for MAS1 and Either NK1 Receptors (NK1R) or GluN1 Subunit in the Lumbar Superficial Dorsal Horn of Naive Mice

(a) Photomicrographs showing fluorescent labeling for MAS1 (green), NK1R (red), and nuclei with DAPI (blue), as well as merged images (right panels). (b) Photomicrographs showing fluorescent labeling for MAS1 (green), GluN1 (red), or nuclei with DAPI (blue), as well as merged images (right panels). (c) Photomicrographs showing fluorescent labeling for MAS1 (green), control for absence of MAS1 labeling with second secondary antibody (Ab) (red), or nuclei with DAPI (blue), as well as merged images (right panels). Scale bar = 100 μ m. (d) Rate of NK1R- or GluN1-positive cells, which express MAS1, in the superficial dorsal horn (laminae I–III). (Color figure can be accessed in the online version.)

preventing the subsequent fluorophore-conjugated anti-rabbit secondary antibody from binding to these sites. To determine whether the Fab fragments could effectively block the antigen binding sites on anti-MAS1 antibodies, we performed a control immunohistochemical labeling in the absence of the primary anti-NK1 or -GluN1 antibodies. As shown in Fig. 3c, the fluorescence produced by the Alexa 568-conjugated secondary antibody completely disappeared suggesting that the Fab fragments effectively blocked the antigen binding sites on anti-MAS1 antibodies. Thus, our results suggest that MAS1 and NK1 or NMDA receptors are expressed in the same cells of the superficial dorsal horn in the spinal cord (Lumbar 5–6). Further, the rate of MAS1-expressed NK1R- or GluN1-positive cells in the superficial dorsal horn (laminae I–III) was 98.8 ± 0.17 or $97.7 \pm 0.36\%$, respectively (Fig. 3d).

DISCUSSION

In the spinal dorsal horn, the activation of NK1 receptors induced by SP leads to an increase in neuronal activity, which occurs *via* NMDA receptor activation.^{12,13} In turn, the latter activation leads to substance P release from primary afferents.¹⁴ Thus, NK1 and NMDA receptors closely interact with each other and their activation plays a facilitative role in the spinal transmission of pain. On the other hand, the activation of the Ang (1–7)/MAS1 axis in the spinal cord can ease several types of pain, including inflammatory¹¹ and neuropathic pain.^{9,10,15} Yet, the effect of Ang (1–7) on the spinal transmission of pain induced by SP or NMDA remains to be demonstrated.

In this study, the i.t. injection of Ang (1–7) attenuated both SP- and NMDA-induced nociceptive behavior while this inhibitory effect was inhibited by the A779, a MAS1 antagonist. These results suggest that Ang (1–7) could attenuate the spinal transmission of pain triggered by the activation of spinal NK1 or NMDA receptors, by acting on MAS1. While the intraplantar injection of formalin is known to cause a biphasic nociceptive response, our recent study has revealed that i.t.-administered Ang (1–7) prevented the second phase without affecting the first phase of the response.¹¹ Similarly, Henry *et al.* have reported that the activation of spinal NK1 receptors is involved only in the second phase of the formalin test.¹⁶ In addition, the knockdown of the spinal GluN1 subunit of the NMDA receptor suppressed the formalin-induced second phase of the nociceptive response, but not the first phase.¹⁷ Taken together, it seems likely that the anti-nociceptive effect of Ang (1–7) in the mouse formalin test results from the inhibition of NK1 or NMDA receptors *via* its binding to spinal MAS1.

From the results of our behavioral experiments, we anticipated that MAS1 co-localized with NK1 and NMDA receptors in order to modulate their activity. Indeed, our immunohistochemical experiments revealed that MAS1 was expressed in both NK1 receptor-positive cells and GluN1-positive cells in the superficial dorsal horn. It has been reported that MAS1 can modulate the activity of other receptors including AT1¹⁸ or AT2 receptors¹⁹ by hetero-oligomerization. Although an interaction between MAS1 and NK1 receptors has not been reported yet, a recent study has revealed that the Ang (1–7)/MAS1 axis exerts an anxiolytic effect by modulating NMDA receptors in the ventral hippocampus.²⁰ Therefore, we suggest that Ang (1–7) attenuates the SP- and NMDA-induced

nociceptive behavior by modulating the activation of their receptors.

In conclusion, we showed that the i.t. injection of Ang (1–7) attenuated the nociceptive behavior induced by SP and NMDA *via* spinal MAS1, which are co-localized with NK1 and NMDA receptors. Since spinal NK1 and NMDA receptors are involved in several types of pain, the activation of spinal Ang (1–7)/MAS1 pathway could represent an effective therapeutic approach for pain management.

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Conflict of Interest The authors declare no conflict of interest.

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