Involvement of descending serotonergic system in antinociception elicited by Kei-Kyo-Zoh-Soh-Oh-Shin-Bu-toh in rats

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(*Received February 27, 1997. Accepted March 21, 1997.*)

Abstract

To confirm the involvement of descending serotonergic system in the analgesic action of the Kampo medicine Kei-Kyo-Zoh-Soh-Oh-Shin-Bu-toh (桂姜棗草黄辛附湯 ; TJ-8023), we examined the effects of intrathecal injections of serotonergic or adrenergic receptor antagonists and a serotonin precursor on the antinociceptive effect of this medicine, using rats with hyperalgesia induced by repeated cold stress. When perorally administered, TJ-8023 (300 mg/kg) produced an apparent inhibition of the hyperalgesia. An intrathecal injection of the serotonin receptor antagonist methysergide (3, 10 and 30 nmol/rat) produced a dose-dependent suppression of the TJ-8023 antinociception. An intrathecal injection of other serotonergic antagonists, cyproheptadine and methiothepin, at a dose of 3 nmol/rat abolished the TJ-8023 antinociception. An intrathecal injection of the serotonin precursor 5-hydroxytryptophan at a dose of 100 nmol/rat, but not at lower doses of 10 and 30 nmol/rat, produced antinociception in rats with hyperalgesia. A combination of an inactive intrathecal dose of 5-hydroxytryptophan (10 nmol/rat) with an inactive peroral dose of TJ-8023 (30 mg/kg) produced an apparent antinociception. An intrathecal injection of the α-adrenoceptor antagonist phentolamine (30 and 100 nmol/rat) did not significantly affect the antinociceptive effect of peroral TJ-8023 (300 mg/kg). The present results suggest the involvement of serotonergic nerve terminals and serotonin 5-HT_1 and/or 5-HT_2 receptors in the spinal cord, probably in the spinal dorsal horn, in the TJ-8023 antinociception and support the idea that this antinociceptive effect is at least partly mediated by the descending serotonergic systems.

Key words Kei-Kyo-Zoh-Soh-Oh-Shin-Bu-toh, analgesia, repeated cold stress, serotonin receptor antagonist, alpha-antagonist, intrathecal, serotonin, 5-hydroxytryptophan.

Abbreviations 5-HT, serotonin ; 5-HTP, 5-hydroxytryptophan ; RCS, repeated cold stress ; i.t., intrathecal ; p.o., peroral ; Kei-Kyo-Zoh-Soh-Oh-Shin-Bu-toh (Kei-Jiang-Zao-Cho-Hang-Xing-Fu-Tang), 桂姜棗草黄辛附湯.

Introduction

Kei-Kyo-Zoh-Soh-Oh-Shin-Bu-toh (桂姜棗草黄辛附湯) is a Kampo medicine used clinically for rheumatism, lumbago, and neuralgia.[1,2] We have shown in animal experiments that the extract of this prescription, TJ-8023, at peroral (p.o.) doses of 30-600 mg/kg inhibits the hyperalgesia of the rat and mouse.[3,4] Especially, hyperalgesia of the rodents induced by repeated cold stress (RCS) was markedly ameliorated by single p.o. administration of TJ-8023 at relatively low doses (30-300 mg/kg), while the same doses were ineffective in naive animals.[3,4] The antinociceptive potency of diclofenac, anti-inflammatory analgesic drug, was similar between naive mice and those with

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RCS-induced hyperalgesia,[5] while it is more effective in rats with inflammatory hyperalgesia than in naive ones.[6] Although hyperalgesia induced by peripheral inflammation is markedly suppressed by intrathecal (i.t.) injection of anti-galanin antibody, RCS-induced hyperalgesia is not affected.[7] Thus, the mechanism of RCS-induced hyperalgesia may be different from those of inflammatory hyperalgesia. The suppression of synaptic transmission mediated by glutamate, substance P and calcitonin gene-related peptide in the spinal cord results in the inhibition of RCS-induced hyperalgesia,[7-9] findings suggesting that spinal mechanisms are at least partly involved in RCS-induced hyperalgesia. As nociceptive transmission in the spinal dorsal horn is inhibited by descending serotonergic and noradrenergic systems,[10,11] we examined the involvement of these monoaminergic systems in the antinociceptive action, especially the inhibition of hyperalgesia induced by RCS. Recently, we have found that the antinociceptive action of TJ-8023 in rats with RCS-induced hyperalgesia is markedly suppressed by an i.t. pretreatment with the serotonergic neurotoxin 5,7-dihydroxytryptamine.[4] Such pretreatment significantly decreased the content of serotonin (5-HT), but not noradrenaline, in the lumbar cord. On the contrary, the antinociceptive action of TJ-8023 was not significantly suppressed by an i.t. injection of the catecholaminergic neurotoxin 6-hydroxydopamine,[4] which depleted lumbar noradrenaline, without changes in lumbar 5-HT. These findings suggest that the descending serotonergic, but not noradrenergic, systems play an important role in the production of the antinociceptive action of this prescription. In these experiments, the i.t. treatment with 5,7-dihydroxytryptamine decreased 5-HT content in the lumbar, but not cervical, cords. However, as the neurotoxin was administered as much as 3 days before the experiments, we could not rule out the possibility that unknown neural changes, secondary to 5-HT depletion, affected the TJ-8023 antinociception. In the present experiments, therefore, in attempting to elucidate further the mechanisms of analgesic action of TJ-8023, especially the involvement of serotonergic terminals and receptors in the spinal cord, we examined the effects of i.t. injections of receptor antagonists and 5-HT precursor on the TJ-8023 antinociception.

Materials and Methods

Animals : Male Sprague-Dawley rats (7 weeks old, SLC, Hamamatsu) were used. They were housed under controlled temperature (24°C) until starting the RCS and light (light on from 08:00 AM to 08:20 PM). Food and water were freely available.

Drugs : TJ-8023 (a gift from Tsumura & Co., Tokyo) was composed of Cinnamomi Cortex (3.0 g), Zingiberis Rhizoma (1.0 g), Glycyrrhizae Radix (2.0 g), Zizyphi Fructus (3.3 g), Ephedrae Herba (2.0 g), Asiasari Radix (2.0 g) and heat-treated Aconiti Tuber (3.5 g). It was suspended in 5% arabic gum (Wako Pure Chemical Ind., Osaka) and perorally (p.o.) administered in a volume of 10 ml/kg. 5-HT receptor antagonists, methysergide maleate (RBI, Natick, USA), cyproheptadine hydrochloride (RBI, Natick, USA) and methiothepin (RBI, Natick, USA), α-adrenoceptor antagonist, phentolamine hydrochloride (Sigma Chem., St. Louis, USA), and 5-HT precursor 5-hydroxytryptophan (5-HTP, RBI, Natick, USA) were dissolved in saline and i.t. injected in a volume of 10 μl through a lumbar puncture between L_3 and L_4 vertebrae, using a stainless-steel needle of 25 gauge. Methysergide, 5-HTP and phentolamine were injected 5 min prior and cyproheptadine and methiothepin were 30 min prior to TJ-8023. Doses are given as the weights of their respective salts.

Repeated cold stress : The animals were exposed to RCS to induce hyperalgesia. The methods for RCS exposure were identical to those described in the previous report,[4] using an automatic RCS apparatus (Hohdensha, Sizuoka). Briefly, rats were exposed to cold environmental temperature (4°C) from 04:30 PM to 10:00 AM and for 30 min every hour between 10:00 AM and 04:30 PM for 3 days, and kept at room temperature (24°C) on the next day, when they were used for analgesic test.

Analgesic test : To measure the nociceptive threshold of the rat for mechanical stimulus, pressure stimulation was applied to the hind paw using a pressure analgesimeter (Ugo Basile, Milan, Italy) with a wedge-shaped piston at a loading rate of 48 g/s.[7] The pressure eliciting struggle or escape behaviors was determined as nociceptive threshold. It was

measured three times before RCS exposure and the injection of receptor antagonists and 5-HTP, and the last data was used as control value before treatment. All the measurements of nociceptive threshold were conducted at a controlled room temperature (24°C). The experiments were conducted according to the ethical guidelines for animal experiments published in Pain.[12]

Statistical analysis : Results were analyzed with repeated measures two-way analysis of variance (RM-ANOVA) or one-way ANOVA, followed by *post hoc* Dunnett's test ; the calculation was done using software SigmaStat (Jandel, San Rafael, USA) and $p < 0.05$ was considered significant. Unless otherwise mentioned, results given are the means and S.E.M. of 6 animals.

Results

Effects of intrathecal 5-HT receptor antagonists and α-adrenoceptor antagonist : The exposure of the rats to RCS produced 33.2 % (S.D.,± 9.0, n = 132) decrease in the nociceptive threshold from 265.2 (S.D., ± 29.8) to 176.3 g (S.D.,± 24.2). All the following experiments were conducted in the rats that had been exposed to RCS. In our previous experiments,[4] TJ-8023 (30, 100 and 300 mg/kg, p.o) produced a dose-dependent antinociception in the RCS-exposed rats, and the most marked and reproducible effect was seen following a dose of 300 mg/kg, without any apparent changes in gross behaviors. In the present experiments, therefore, we examined the effects of serotonergic and adrenergic antagonists on the antinociceptive action of TJ-8023 in a p.o. dose of 300 mg/kg.

When i.t. pretreated, methysergide (3, 10 and 30 nmol/rat) produced a dose-dependent inhibition of the antinociceptive action of TJ-8023 (300 mg/kg, p.o.) in the RCS rats (Fig. 1) : application of RM-ANOVA demonstrated the significant main effect of methysergide [$F(3,20) = 7.77$, $p < 0.01$] and group × time interaction [$F(9,60) = 7.21$, $p < 0.0001$]. It is noteworthy that methysergide at the highest dose tested (30 nmol/rat) completely suppressed the TJ-8023 antinociception.

The effects of pretreatment with two other 5-HT receptor antagonists on the TJ-8023 antinociception

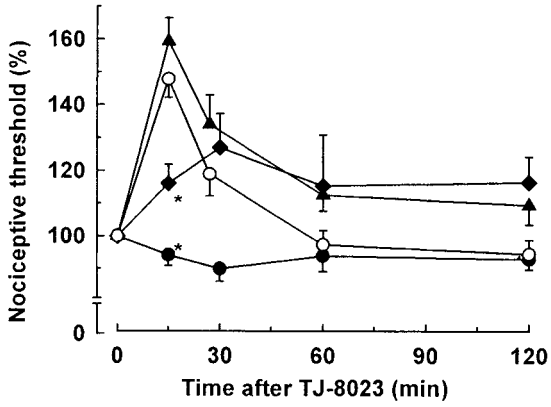

Fig. 1 Effects of intrathecal pretreatment with methysergide on the antinociceptive action of TJ-8023 in rats exposed to repeated cold stress. TJ-8023 (300 mg/kg, p.o.) was administered 5 min after an intrathecal injection of vehicle (○) or methysergide at doses of 3 (▲), 10 (◆) and 30 nmol/rat (●), $*p < 0.05$ when compared with vehicle. Values are the means and S.E.M. of 6 animals.

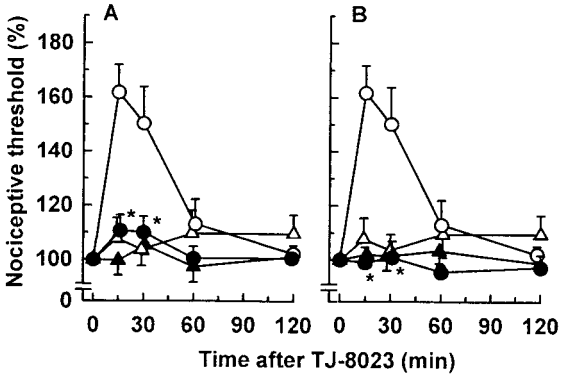

Fig. 2 Effects of intrathecal pretreatment with cyproheptadine (A) or methiothepin (B) on the antinociceptive action of TJ-8023 in rats exposed to repeated cold stress. TJ-8023 (300 mg/kg, p.o.) was administered 30 min after an intrathecal injection of cyproheptadine (3 nmol/rat), methiothepin (3 nmol/rat) or vehicle. ○, TJ-8023 after intrathecal vehicle ; ●, TJ-8023 after either cyproheptadine or methiothepin ; △ vehicle (p.o.) after intrathecal vehicle ; ▲, vehicle (p.o.) after either cyproheptadine or methiothepin. $*p < 0.05$ when compared between intrathecal antagonists- and vehicle-treated groups that were given TJ-8023. Values are the means and S.E.M. of 6 animals.

were shown in Fig. 2. The antinociceptive action of TJ-8023 (300 mg/kg, p.o.) was nearly abolished by i.t. pretreatment with cyproheptadine at a dose of 3 nmol/rat (Fig. 2A) ; the application of RM-ANOVA to two groups given TJ-8023 demonstrated significant

main effect of cyproheptadine [$F(1,10)=13.5$, $p<0.01$] and group×time interaction [$F(3,30)=17.7$, $p<0.0001$]. Similarly, the antinociception of TJ-8023 was completely suppressed by i.t. pretreatment with methiothepin at a dose of 3 nmol/rat (Fig. 2B) ; RM-ANOVA of two groups given TJ-8023 revealed the significant main effect of methiothepin [$F(1,10)=14.1$, $p<0.01$] and group×time interaction [$F(3,30)=14.0$, $p<0.0001$].

The antinociceptive action of TJ-8023 (300 mg/kg, p.o.) was not significantly [$F(2,15)=2.19$, $p>0.05$] affected by i.t. pretreatment with the α-adrenoceptor antagonist phentolamine (30 and 100 nmol/rat). The nociceptive threshold at 15 min after TJ-8023 administration was 232.5±5.6, 272.5±19.8, and 251.5±11.2 g (n=6 each group) in rats pretreated with vehicle, 30 and 100 nmol/rat of phentolamine, respectively, while the threshold of rats given p.o. vehicle after phentolamine (30 nmol/rat, i.t.) was 184.0±10.0 g (n=6).

Effects of intrathecal 5-hydroxytryptophan : An i.t. injection of the serotonin precursor 5-HTP in a dose of 100 nmol/rat increased the nociceptive threshold, without significant effects at lower doses of 10 and 30 nmol/rats ; the effects at the dose of 100 nmol/rat appeared by 10 min, peaked at 10-30 min and subsided by 60 min (Fig. 3). To elucidate the involvement of serotonergic terminals present in the spinal cord in the

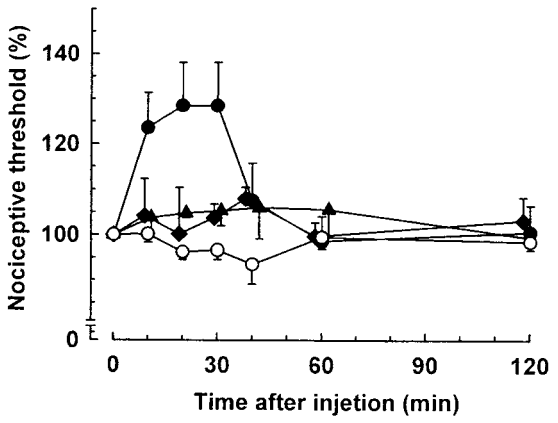

Fig. 3 Effects of intrathecal injections of the serotonin precursor 5-hydroxytryptophan (5-HTP) on the nociceptive threshold of rats exposed to repeated cold stress. The rats were intrathecally given vehicle (○) or 5-HTP at doses of 10 (▲), 30 (◆) and 100 nmol/rat (●). Values are the means and S.E.M. of 6 animals.

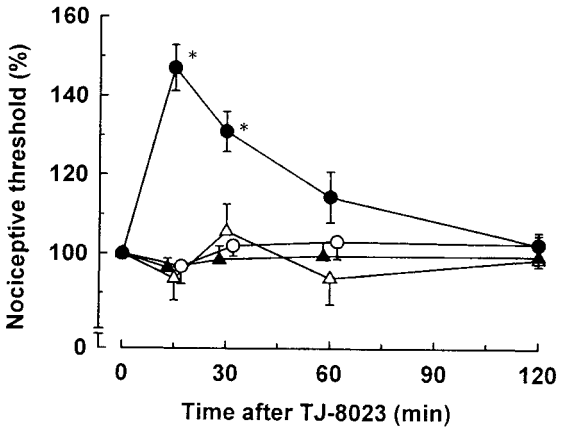

Fig. 4 Enhancement of antinociceptive action of TJ-8023 by an intrathecal injection of 5-hydroxytryptophan (5-HTP) in rats exposed to repeated cold stress. 5-HTP (10 nmol/rat) was intrathecally given 5 min prior to p.o. administration of TJ-8023 (30 mg/kg). △, vehicle (p.o.) after intrathecal vehicle ; ▲, vehicle (p.o.) after intrathecal 5-HTP ; ○, TJ-8023 after intrathecal vehicle ; ●, TJ-8023 after intrathecal 5-HTP. *$p<0.05$ when compared between intrathecal 5-HTP- and vehicle-treated groups that were given TJ-8023. Values are the means and S.E.M. of 6 animals.

antinociceptive effect of TJ-8023, we examined whether an i.t. injection of 5-HTP would enhance the TJ-8023 action. Although TJ-8023 (30 mg/kg, p.o.) exerted no effect alone on the nociceptive threshold, it produced a marked antinociceptive effect following i.t. pretreatment with 5-HTP at a dose of 10 nmol/rat (Fig. 4) ; the effect of a combination of TJ-8023 with 5-HTP was significantly [Dunnett's test after RM-ANOVA] different from that of either TJ-8023 or 5-HTP alone. The peak effect was similar to that at a dose of 300 mg/kg (see Figs. 1 and 2).

Discussion

In the present experiments, we demonstrated that antinociceptive action of TJ-8023 was inhibited by i.t. injections of three different serotonergic receptor antagonists, findings suggesting the involvement of spinal serotonergic receptors. In our previous studies using the paw-pressure test, we found that the antinociceptive action of TJ-8023 diminished after i.t. pretreatment with the serotonergic neurotoxin 5,7-dihydroxytryptamine.[4] Such treatment selectively decreased the content of 5-HT in the lumbar cord

without any apparent changes in the cervical content.[4] Considering that nociceptive signals from the periphery are mainly conveyed to the dorsal horn, especially the superficial dorsal horn, an important role of serotonergic terminals in the dorsal horn is suggested. To further confirm this view, we examined the effect of i.t. injection of the 5-HT precursor 5-HTP and found that such treatment enhanced the TJ-8023 action, suggesting the involvement of serotonergic nerve terminals in the spinal cord. One possible explanation of the mechanisms of the synergistic effect of 5-HTP on the TJ-8023 action is that TJ-8023 activated the descending serotonergic systems to inhibit nociceptive transmission in the spinal dorsal horn and that 5-HTP pretreatment increased the release of 5-HT following TJ-8023 administration. If so, RCS-induced hyperalgesia might not be due to a decrease in the activity of the descending serotonergic systems. This view is consistent with the findings that TJ-8023 at doses examined was effective in rodents with hyperalgesia induced by RCS but not in naive animals.[3,4] In this context, it is noteworthy that antinociceptive potency of morphine was less in RCS rats than in naive ones and that the morphine effect was markedly suppressed by intrathecal pretreatment with catecholaminergic neurotoxin rather than with serotonergic neurotoxin in either naive[13] or RCS-exposed rats.[4] These findings taken together strongly suggest that the descending serotonergic systems and serotonergic synapses in the dorsal horn play an important role in the production of antinociceptive action of this Kampo medicine at least in the rats with hyperalgesia induced by RCS.[4]

In the present experiments, three serotonergic receptor antagonists, that is, methiothepin, methysergide and cyproheptadine, inhibited the TJ-8023 effect. Methiothepin has substantial affinities for both 5-HT_1 and 5-HT_2 receptors and the other two have high affinity for 5-HT_2 receptors.[14] However, as the effective concentrations of these antagonists (3-30 nmol/10 μl, i.t.) were relatively high, the present data could not determine the serotonergic receptor subtypes responsible for the TJ-8023 effect. It is well established that the descending serotonergic systems that originate from the caudal raphe nuclei, especially nucleus raphe magnus, inhibit nociceptive transmission in the spinal dorsal horn.[11,13,15-17] With regard to 5-HT_1 receptors, fifty percent of nociceptive dorsal horn neurons inhibited by the stimulation of the nucleus raphe magnus are also inhibited by iontophoretic application of selective 5-HT_{1A} agonist.[18] Iontophoretic application of 5-HT_{1A} agonist inhibits nociceptive responses of the dorsal horn neurons, which are inhibited also by iontophoretic 5-HT_{1B} agonist.[19] Autoradiographic receptor mapping studies have revealed the localization of 5-HT_{1A} and 5-HT_{1B} receptors in regions that receive nociceptive inputs from the periphery ; 5-HT_{1A} receptors are localized in the superficial laminae I and II and 5-HT_{1B} receptors are widespread throughout the dorsal horn with high densities in lamina X.[20,21] In addition, i.t. injections of 5-HT_{1A} and 5-HT_{1B} receptor agonists produce behavioral antinociception in rodents.[22-25] On the other hand, with regard to 5-HT_2 receptors, an i.t. injection of 5-HT_2 receptor agonist does not produce behavioral antinociception.[23] 5-HT_2 receptors are present more densely in the ventral horn than in the dorsal horn.[21] These electrophysiological, behavioral and anatomical studies taken together suggest that spinal 5-HT analgesia is mediated by 5-HT_1 receptors rather than 5-HT_2 receptors and raise the possibility that the TJ-8023 antinociceptive effects are at least in part mediated by 5-HT_1 receptors in the spinal dorsal horn.

The primary site of the antinociceptive action of TJ-8023 remains obscure. In the present experiments, the TJ-8023 effect peaked at 15 min after the p.o. administration, which was the same as our previous experiments.[3,4] One possible explanation for such early peak time (and onset) of the TJ-8023 antinociceptive action is that this Kampo medicine acted primarily on the periphery rather than on the central nervous system. In this context, the stimulation of vagal afferents modulates the spinal nociceptive reflex[26,27] and nociceptive responses of spinal dorsal horn neurons.[28,29] Such vagus-associated modulation of nociceptive responses is at least partly mediated by the descending serotonergic systems.[27,30] From these findings we speculate that this Kampo acted primarily on the periphery, for example gastrointestinal tract, to activate the descending serotonergic system via vagal afferents. Recently, we have

found that mao-bushi-saishin-toh shows antinociceptive effects similar to that of TJ-8023 ; it produced marked antinociception in rats with RCS - induced hyperalgesia, without effects in naive ones, and that such effect was also inhibited by suppression of serotonergic functions in the spinal cord (unpublished observation). Experiments for determining the involvement of vagus functions in these Kampo medicines are now conducted in our laboratory.

Conclusions

We demonstrated that the antinociceptive effect of peroral administration of Kei-Kyo-Zoh-Soh-Oh-Shin-Bu-toh (TJ-8023) was inhibited by an i.t. injection of three serotonergic antagonists and enhanced by that of serotonin precursor 5-HTP. These results suggest an important role of the serotonergic nerve teriminals and serotonin receptors in the spinal cord, probably in the dorsal horn, in the antinociceptive effect of TJ-8023 and support the idea that this effect is at least partly mediated by the descending serotonergic systems.

Acknowledgments

We thank Prof. K. Terasawa of Toyama Medical and Pharmaceutical University for making RCS apparatus available for us. We also thank Tsumura & Co. for the supply of TJ-8023. A portion of this study was supported by a Grant-in-Aid for Scientific Research (B) (No.07557379).

和文抄録

漢方方剤桂姜棗草黄辛附湯（TJ-8023）の鎮痛作用への下行性セロトニン神経系の関与を確証する目的で，反復低温ストレスにより痛覚過敏を惹起したラットを用い，セロトニン受容体拮抗薬，アドレナリン受容体遮断薬及びセロトニン前駆体を脊髄クモ膜下腔内注射して，この方剤の抗侵害受容作用に対する作用を調べた。TJ-8023 (300 mg/kg) が経口投与により，この痛覚過敏を明らかに抑制した。セロトニン受容体拮抗薬 methysergide (3, 10, 30 nmol/rat) の脊髄クモ膜下腔内注射が，TJ-8023 のこの抗侵害受容作用を用量依存的に抑制した。他のセロトニン受容体拮抗薬 cyproheptadine と methiothepin は，3 nmol/rat の用量の脊髄クモ膜下腔内注射で TJ-8023 の抗侵害受容作用を消失した。セロトニン前駆体 5-hydroxytryptophan の脊髄クモ膜下腔内注射が，100 nmol/rat の用量で痛覚過敏ラットに抗侵害受容作用を発現したが，10 及び 30 nmol/rat では無効であった。無効量の 5-hydroxytryptophan (10 nmol/rat) の脊髄クモ膜下腔内注射と無効量の TJ-8023 (30 mg/kg) の経口投与の組合せが，明らかな抗侵害受容作用を発現した。α-アドレナリン受容体拮抗薬 phentolamine (30, 100 nmol/rat) の脊髄クモ膜下腔内注射は，TJ-8023 (300 mg/kg) の経口投与の抗侵害受容作用に有意な影響を及ぼさなかった。本結果は，TJ-8023 の抗侵害受容作用に脊髄（おそらく脊髄後角）のセロトニン神経終末とセロトニン 5-HT_1 あるいは 5-HT_2 受容体が関与することを示唆し，この抗侵害受容作用に少なくとも一部下行性セロトニン神経系が関与するとの考えを支持するものである。

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