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ORIGINAL ARTICLE

The antinociceptive effect of intrathecal tramadol in rats: the role of alpha 2-adrenoceptors in the spinal cord

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Abstract
Purposes The alpha 2 (α_2)-adrenoceptor is highly important in the antinociception of tramadol administered systemically and intrathecally. However, it is unclear whether tramadol at the spinal level exerts an antinociceptive effect by directly binding with α_2-adrenoceptors in the spinal cord. This study was conducted to investigate the relationship between α_2-adrenoceptors and the antinociception of tramadol at the spinal level.
Methods The rat formalin test was designed to determine whether the intrathecal α_2-adrenoceptor antagonist yohimbine could reverse the antinociceptive effect of intrathecal tramadol. The binding affinity of tramadol for α_2-adrenoceptors in the spinal cord was determined by radioligand binding assay using the labeled α_2-adrenoceptor antagonist [^{3}H]-yohimbine.
Results The nociceptive test showed that intrathecal tramadol induced significant antinociception whereas pretreatment with intrathecal yohimbine partially reversed this antinociception. Scatchard analysis of the binding data showed [^{3}H]-yohimbine had high affinity (K_d = 1.79 nM) for the α_2-adrenoceptor in the rat spinal cord, and that tramadol inhibited specific binding of [^{3}H]-yohimbine with the spinal cord membranes with a high affinity constant (K_i = 34.14 μM) and an IC50 of 68.25 μM, which indicated that tramadol was much less potent than [^{3}H]-yohimbine at binding with α_2-adrenoceptors of the spinal cord.
Conclusion The results suggested that, with very weak binding affinity for α_2-adrenoceptors, the antinociception of intrathecal tramadol is partially related to α_2-adrenoceptors, and its intrathecal antinociception may mainly involve its indirect activation of α_2-adrenoceptors in the spinal cord.

Keywords Tramadol · Yohimbine · Spinal cord · Antinociception · α_2-Adrenoceptor · Affinity

Introduction

Tramadol is a unique, centrally acting analgesic drug which is mainly used for treatment of moderate to severe pain [1]. The antinociceptive action of tramadol is attributed to two mechanisms—in addition to the μ-opioid agonist effect, tramadol can exert a modulatory effect on the central monoaminergic pathways to inhibit the neuronal uptake of noradrenaline and serotonin (5-HT) [2–5]. Recently, in addition to the intravenous route, tramadol has been widely used for clinical pain relief through the epidural, caudal, and even intrathecal routes [6–8]. However, the exact mechanisms of action of tramadol at the spinal level are not completely understood.

Animal and clinical studies have shown that intravenous administration of the alpha 2 (α_2)-adrenoceptor antagonist yohimbine partially antagonizes the antinociceptive effect of orally or intravenously administrated tramadol [9, 10]. Investigation has revealed that the antinociceptive effect of tramadol administered intrathecally was significantly attenuated by treatment with either yohimbine or ritanserin

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administered intraperitoneally [2]. These findings indicated that the α_2-adrenoceptor is, at least in part, highly important in the antinociception of tramadol administered systemically and intrathecally. However, it is not clear whether tramadol at the spinal level exerts its antinociceptive effect by directly binding with α_2-adrenoceptors in the spinal cord. The objective of this study was to investigate the relationship between the α_2-adrenoceptor and the antinociception of tramadol at the spinal level, by use of the rat formalin test, and to determine the binding affinity of tramadol with α_2-adrenoceptors in the spinal cord by radioligand binding essay.

Materials and methods

Animal preparation

This study was carried out using a procedure approved by Institutional Animal Care Committee of Sun Yat-sen University, China. The animals were housed in plastic boxes with ad libitum food and water in a colony room under natural light. All testing was conducted between 09:00 and 17:00 hours. First the rats received an indwelling intrathecal catheter for later spinal drug delivery during pentobarbital (50 mg/kg, intraperitoneally) anesthesia, and then a polyethylene-10 catheter was advanced 8.5 cm caudally through an incision in the atlantooccipital membrane into the subarachnoid space extending to the level of the rostral lumbar enlargement. The external end of the catheter was tunneled subcutaneously with the exit at the top of head and plugged with a piece of steel wire. The skin was sealed with 3-0 silk sutures and the rats were placed in individual cages for recovery. Only animals with no evidence of neurological deficits after catheter insertion were chosen for study, and the behavioral testing started 4–5 days after intrathecal catheter implantation.

Experimental procedure

The antinociceptive experiment was designed to determine whether the intrathecal α_2-adrenoceptor antagonist yohimbine could reverse the antinociceptive effect of intrathecal tramadol. Thirty-two adult male Wistar rats weighing 280–320 g, with intrathecal catheter, were randomly allocated into four groups ($n = 8$) for treatment with different intrathecal drugs: normal saline (control group), tramadol (10 μg) group, yohimbine (10 μg) group, and pretreatment group (10 μg yohimbine + 10 μg tramadol). The dose of tramadol came from a previous study in which intrathecal administration of tramadol (10 μg) induced significant antinociception in the rat formalin test [11]. The dose of yohimbine was determined in a preliminary experiment in which 10 μg yohimbine significantly reversed the antinociception of tramadol. The groups of rats initially received an injection of formalin in the hind paws and the drugs were administered intrathecally 15 min later. For the pretreatment group, tramadol was administered 5 min after administration of yohimbine.

Nociceptive test

Formalin was used for the nociceptive test on all the four groups of rats mentioned above. Animals were placed individually in Plexiglas testing chambers (30 × 30 × 30 cm) and left to acclimate for at least 60 min. One mirror was situated behind the chamber and another one was located at a 45° angle below the floor of the chamber to enable unobstructed viewing of the rat's paws. After acclimation, the rats were given a subcutaneous injection of 50 μL 2.5% formalin solution into the plantar surface of the left hind paw using a 30-G needle, and were then returned to the testing chambers. Observation of the rat behavior started 30 min after the formalin injection and lasted 30 min, which was divided into six blocks of 5 minutes. Categorization of the behavior was as originally described by Dubuisson et al. and reiterated by Abbott et al. as "0 = normal weight bearing on the injected paw, 1 = limping during locomotion or resting the paw lightly on the floor, 2 = elevation of the injected paw so that at most the nails touch the floor, and 3 = licking, biting or shaking the injected paw" [12, 13]. A weighted pain score was calculated for each treated rat at each 5-min observation period by multiplying the amount of time spent in each category by the assigned category weight, then summing these products and dividing the sum by 5 min. The researcher responsible for assessing the behavioral test was unaware of the experimental circumstances of each rat, and no formalin test was repeated on any rats.

Drugs and administration method

The drugs used in this study were tramadol (Gruenenthal, Germany) and yohimbine hydrochloride (Sigma, St Louis, USA). [^{3}H]-Yohimbine (specific activity 79.2 Ci/mmol) was purchased from New England Nuclear, Boston, MA, USA. Tramadol and yohimbine solutions were freshly prepared in dimethyl sulfoxide (DMSO) and normal saline, respectively, adjusting to pH 6.8–7.1 and filtering before administration. These agents were intrathecally administered in 10 μL solution followed by an additional 10 μL of normal saline to flush the catheter using a hand-driven, gear-operated syringe pump.

Membrane binding experiments in the spinal cord

Sixteen rats different from the 32 rats used as described above for nociceptive test were sacrificed by decapitation,

the lumbosacral spinal cords were quickly removed, and the protein content determined as described elsewhere [14]. In brief, the dorsal half was dissected and used for the binding experiment. The dorsal tissue was thawed, quickly chopped, suspended in ice-cold 50 mM Tris buffer containing 3 mM $MgCl_2$ and 1 mM EGTA (pH 7.4), and subsequently homogenized and disrupted by sonication. The homogenate was then centrifuged at 5,000 rpm for 10 min at 4°C, and the supernatant was collected and centrifuged at 48,000 rpm for another 20 min at 4°C. The pellet obtained was resuspended in fresh Tris buffer, centrifuged once again, and the final pellet was separated and resuspended in 50 mM Tris buffer containing 3 mM $MgCl_2$, 100 mM NaCl, and 0.2 mM EGTA (pH 7.7) to be disrupted by sonication for 5 s. The protein content of the last tissue sample was measured by Bradford's method using bovine serum albumin as standard (Protein Assay Kit II; Bio-Rad Laboratories, Hercules, CA, USA).

Saturation radioligand binding experiments were performed on 100 μL tissue samples prepared from spinal cord membranes as mentioned above. The samples were incubated with different concentrations of [^{3}H]-yohimbine solution (0.18–4.5 nM) in the absence or presence of 1 μM unlabeled yohimbine to determine the total and nonspecific α_2-adrenoceptor binding, respectively, of yohimbine with the spinal cord membranes. The samples were incubated in duplicate in Tris buffer at 25°C for 60 min, and the reaction was terminated by filtrating through Whatman GF/B filters on a cell harvester with cold Tris buffer (pH 7.4). The filters were then immersed in the scintillation fluid and incubated overnight at room temperature. Next day the radioactivity of the scintillation fluid was quantified using a liquid scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA, USA) to determine the concentrations of free and bound [^{3}H]-yohimbine.

To determine the inhibitive effect of tramadol on the specific binding of [^{3}H]-yohimbine with the spinal cord membranes, competitive binding experiments were performed on 100 μL tissue samples using 10 different concentrations of tramadol in the range 0.21 nM–2.1 mM and a fixed concentration of [^{3}H]-yohimbine (1.8 nM). The tissue samples were incubated in [^{3}H]-yohimbine solution combined with tramadol solutions of different concentrations. Following the above procedure, the incubated samples were treated and tested for the concentration of bound [^{3}H]-yohimbine in the presence of tramadol.

Data analysis

The weighted pain scores were determined for each 5-min interval after injection of formalin, and the results were expressed as mean ± SD for the group of rats ($n = 8$) in that 5-min interval. Statistical analysis among multiple groups for the same 5-min interval was performed using one-way ANOVA followed by the Bonferroni post hoc test. $P < 0.05$ was regarded as statistically significant.

The concentrations of free and bound [^{3}H]-yohimbine obtained from the radioligand binding experiments were analyzed by the method of Scatchard using nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA, USA), and the maximum specific binding (B_{max}) and the dissociation constant (K_d) were calculated from the saturation binding profiles. The results were means from six replicate experiments.

IC_{50}, the concentration of unlabelled ligand (tramadol) which caused inhibition of 50% of binding of [^{3}H]-yohimbine during competition experiments, was determined from the displacement curves using nonlinear regression (Prism; GraphPad Software). The inhibition constant K_i was calculated from the IC_{50} value by means of the Cheng and Prusoff equation, $(K_i) = IC_{50}/(1 + L/K_d)$, where L and K_d are the concentration and affinity of the radiolabeled ligand ([^{3}H]-yohimbine) [15]. The results were means from ten experiments.

Results

The antinociceptive effect of tramadol

On the basis of the nociceptive test, the weighted pain scores were calculated for each treated group of rats during the 30 min of observation; the results are plotted in Fig. 1.

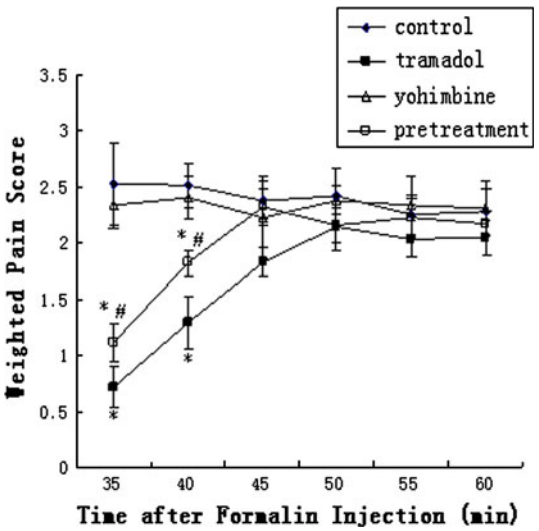

Fig. 1 Effects of intrathecal administration of saline (control), tramadol, yohimbine, and tramadol combined with yohimbine (pretreatment) on weighted pain score evoked by injection of formalin. Results are expressed as mean ± standard deviation, $n = 8$. Compared with the control group, $^*P < 0.01$; compared with the tramadol group, $^{\#}P < 0.05$

It was found that the pain scores of the tramadol group were significantly lower than those of the control group 35 min (0.71 ± 0.20 vs. 2.53 ± 0.37) and 40 min (1.29 ± 0.19 vs. 2.51 ± 0.20) after injection of formalin ($P < 0.01$). There was no significant difference for the pain scores between the yohimbine and control groups during the whole observation ($P > 0.05$). For the yohimbine pretreatment group, the pain scores were significantly higher than those of the tramadol group 35 min (1.11 ± 0.18 vs. 0.71 ± 0.20) and 40 min (1.82 ± 0.24 vs. 1.29 ± 0.19) after injection of formalin ($P < 0.05$), but still lower than scores of the control group ($P < 0.01$). This suggested that intrathecal administration of yohimbine might partially reverse the antinociception of tramadol at the spinal level.

α_2-Adrenoceptor binding of [^{3}H]-yohimbine

In accordance with the method of Rosenthal [16], the concentrations of free and bound [^{3}H]-yohimbine obtained from the radioligand binding experiments were analyzed by the Scatchard method and a straight line was obtained, as shown in Fig. 2, to indicate the saturable binding of [^{3}H]-yohimbine with the spinal cord membranes and the presence of a single class of [^{3}H]-yohimbine-binding sites. The affinity constant K_d and the maximum specific binding B_{max} of [^{3}H]-yohimbine with the spinal cord membranes were estimated to be 1.79 nM and 58.47 fmol/mg protein, respectively.

α_2-Adrenoceptor binding of tramadol in competitive experiments

The concentration of bound [^{3}H]-yohimbine in the presence of different concentrations of tramadol is shown in Fig. 3. The results illustrate that the inhibitive effect of tramadol on the specific binding of [^{3}H]-yohimbine with the spinal cord membranes only appeared at very high tramadol concentrations. The calculated K_i value of 34.14 μM and IC_{50} of 68.25 μM indicated a weak affinity of tramadol for the spinal α_2-adrenoceptors, and slight inhibition of the binding of yohimbine. Compared with the K_d of 1.79 nM for [^{3}H]-yohimbine, this demonstrated that tramadol, with K_i of 34,140 nM, was much less potent in binding with the spinal α_2-adrenoceptor.

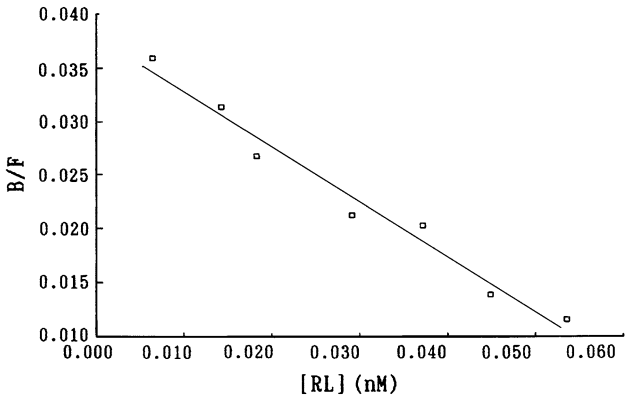

Fig. 2 Scatchard analysis of specific binding data of [^{3}H]-yohimbine with rat spinal cord membranes. Results are expressed as means, $n = 6$. *RL* is the concentration of bound [^{3}H]-yohimbine, and *B/F* is the ratio of bound [^{3}H]-yohimbine to free [^{3}H]-yohimbine. The *line* indicates the saturable binding of [^{3}H]-yohimbine with the spinal cord membranes. The *square boxes* are the B/F in the presence of different RL

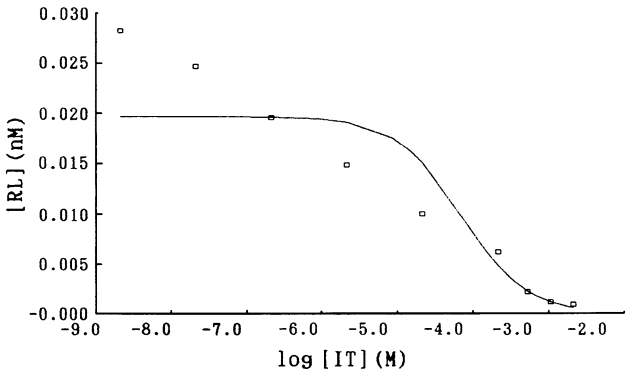

Fig. 3 Binding of [^{3}H]-yohimbine to rat spinal cord membranes under competitive inhibition by tramadol. Results are expressed as means, $n = 10$. *IT* denotes tramadol and *RL* denotes bound [^{3}H]-yohimbine. The curved line shows the inhibitive effect of tramadol on the specific binding of [^{3}H]-yohimbine with the spinal cord membranes. The *square boxes* represent the concentrations of bound [^{3}H]-yohimbine in the presence of different concentrations of tramadol

Discussion

As reported previously, intravenous administrations of yohimbine and idazoxan partially antagonized the antinociception of intravenously administered tramadol in arthritic rats [3]. It has also been observed, by monitoring subjective and objective pain thresholds, that intravenously administered yohimbine partially reverses the analgesic effect of orally administered tramadol in volunteers [9]. However, there are no reports of the effects of intrathecally administered α_2-adrenoceptor antagonists on the antinociception of tramadol at the spinal level. Therefore, in this study, intrathecal administration of yohimbine was used to investigate the effect of this α_2-adrenoceptor antagonist on the antinociception of tramadol at the spinal level, by use of the rat formalin test.

The formalin test is thought to be a more valid model for clinical pain assessment than classic tests using mechanical or heat stimuli because its biphasic nociceptive response (phase 1, 0–10 min, acute pain; phase 2, 21–60 min, tonic pain) seems to be closely related to tissue injury [11, 12]. In

this study, the formalin test was used for the first time to evaluate the antinociception of tramadol at the spinal level. As the study was focused on investigation of the effect of the spinal α_2-adrenoceptor antagonist yohimbine on the antinociception of intrathecal tramadol, only behavioral changes of rats from 30 to 60 min after the formalin injection were observed and evaluated for pain score calculation.

On the basis of comparison of the pain scores for four study groups in this nociceptive study, it was demonstrated that intrathecal tramadol could exert significant antinociceptive effect after injection of formalin, and that pretreatment with intrathecal yohimbine could partially reverse the antinociception of tramadol. This suggested that the antinociception of tramadol at the spinal level may partially involve spinal α_2-adrenoceptors, in accordance with other experiments using the tail-flick and hot-plate assays [2, 17].

Our results also suggest there may be other mechanisms, besides the α_2-adrenoceptor effect, affecting the antinociception of tramadol at the spinal level. It has already been demonstrated that tramadol has less affinity for the μ-opioid receptor than morphine and only 40% antinociception of tramadol was antagonized by naloxone [2], indicating that another non-opioid mechanism might contribute to the overall analgesic effect of tramadol. Moreover, it has been reported that the antinociceptive effect of tramadol was significantly diminished in 5-HT-lesioned mice, and intrathecal injection of 5-HT receptor antagonists blocked tramadol-induced antinociception, indicating that the descending serotonergic pathways and spinal 5-HT receptors are of crucial importance in the antinociceptive effects of tramadol [18]. These findings, together with our results, suggest that the dual mechanisms proposed in previous studies [2–5] may be relevant to the antinociception of tramadol at the spinal level, and α_2-adrenoceptors may be important in modulation of the antinociception of tramadol at the spinal level. To further evaluate the exact involvement of spinal α_2-adrenoceptors in the antinociception of tramadol, the binding affinity of tramadol with α_2-adrenoceptors in the spinal cord was investigated by conducting binding studies.

Scatchard analysis of the binding data showed [^{3}H]-yohimbine had high affinity ($K_d = 1.79$ nM) for the α_2-adrenoceptor in the rat spinal cord, and, therefore, it was feasible to use yohimbine as an α_2-adrenoceptor antagonist to investigate the mechanisms of antinociception of tramadol. The results of the competitive binding study showed that tramadol had quite weak binding affinity ($K_i = 34$, 140 nM) for α_2-adrenoceptors in the spinal cord, indicating that tramadol might not directly activate the spinal α_2-adrenoceptor to produce its antinociception at the spinal level. The relationship between the antinociception of tramadol and α_2-adrenoceptors at the spinal level may be related to two factors. First, it has been demonstrated that tramadol can enhance the extraneuronal free noradrenaline level in the spinal cord by competitive interference with the noradrenaline uptake mechanism [19]. Second, several anatomical, electrophysiological and biochemical studies have shown that noradrenaline produces its antinociceptive effect by activating α_2-adrenoceptors [20–22]. Therefore, it can be deduced that intrathecal tramadol may produce its antinociception by elevating the noradrenaline level, thereby activating spinal α_2-adrenoceptors. Nevertheless, further study is necessary to furnish direct evidence (e.g. norepinephrine level is up-regulated after administration of tramadol at the spinal level) to strengthen the above conclusion. In addition, a study [23] has shown that tramadol produced outward currents by activating μ-opioid receptors, but not α_2-adrenoceptors, in rat spinal cord neurons, which also suggests that tramadol might not directly activate the spinal α_2-adrenoceptor to produce its antinociception at the spinal level.

In conclusion, these findings demonstrate that tramadol produces antinociception at the spinal level and has very weak binding affinity for α_2-adrenoceptors in the spinal cord. Its antinociception at the spinal level may be mainly attributed to its indirect activation of spinal α_2-adrenoceptors.

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