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Interaction between midazolam and epibatidine in spinally mediated antinociception in rats

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Abstract
Purpose. Both γ-aminobutyric acid $(GABA)_A$ receptors and nicotinic cholinergic receptors have important roles in antinociception in the spinal cord. The antinociceptive effects of midazolam (a $GABA_A$ agonist) and epibatidine (a nicotinic cholinergic agonist) in the spinal cord have been reported. The present study was performed to investigate the interaction between intrathecal midazolam and epibatidine.
Methods. Sprague-Dawley rats with lumbar intrathecal catheters were tested for their tail withdrawal response to thermal stimulation (tail-flick test) or for their paw flinches in response to formalin injection (formalin test) after the intrathecal administration of epibatidine or a combination of midazolam and epibatidine. The combination doses were 1/16, 1/8, 1/4, 1/2, 1, 2, and 4 times the 50% effective dose (ED50) of each agent in each test. The interaction of midazolam and epibatidine was investigated by isobolographic analysis. Behavioral side effects were also investigated.
Results. In the tail-flick test, the ED50 values of the combination were significantly higher than the theoretical additive values. In the formalin test, the ED50 values of the combination were significantly lower than the theoretical additive values in phase 1, but were not different from the theoretical additive values in phase 2.
Conclusion. The intrathecal combination of midazolam and epibatidine had antagonistic effects on thermal acute nociception, while the combination had synergistic effects on acute inflammatory nociception, with only additive effects on inflammatory-facilitated nociceptive responses.

Key words Antinociception · Gamma-aminobutyric $acid_A$ receptor · Nicotinic cholinergic receptor · Spinal cord

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Introduction

Midazolam administered intrathecally induces antinociception through γ-aminobutyric acid $(GABA)_A$ receptors coupling with benzodiazepine receptors in the spinal cord [1]. In a rat experiment, intrathecally administered midazolam exerted antinociception in thermally induced acute nociception and in formalin-induced acute and facilitated nociceptive responses [2]. Midazolam has also shown analgesic effects in human studies of intrathecal [3] and epidural administration [4].

Nicotine is well known to produce a potent antinociceptive effect [5]. Nicotinic cholinergic receptors in the spinal cord seem to have an important role in the modulation of persistent pain [6]. However, the antinociceptive effects of nicotine are seen with high and near-toxic doses [7], which prevents nicotine from being used clinically. Epibatidine is a highly potent but nonselective stimulant of neuronal nicotinic acetylcholine receptors [8] and has a potent analgesic effect [7]. Spinally administered epibatidine inhibits the development of hyperalgesia and inflammation [6]. In our previous studies, intrathecally administered epibatidine showed dose-dependent antinociception in thermally induced acute nociception [9]. However, epibatidine is also toxic at doses slightly higher than those required for antinociception [10], although it is safer than nicotine.

The interaction between two different receptor mechanisms might be expected to enhance antinociception, with a reduction in the toxic effect of the agents acting on each receptor. However, there is no study to show antinociceptive interaction between $GABA_A$ receptors and nicotinic cholinergic receptors in the spinal cord. The purpose of the present study was to investigate the interaction of spinally administered midazolam and epibatidine in two different nociceptive models in rats (acute thermal nociception [tail-flick test] and inflammatory-mediated nociception [formalin test]).

Materials and methods

After the approval of the Research Committee of the University of Tokyo was obtained, male Sprague-Dawley rats (280–300 g; Nippon Bio-Supply, Tokyo, Japan) were implanted with lumbar intrathecal catheters under halothane (2 %) in 100% oxygen inhalation. An 8.5-cm polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted caudally to the thoracolumbar level in the intrathecal space through the atlanto-occipital membrane. The rostral part of the catheter was plugged with a 28-gauge (G) steel wire and put through to the top of the skull. Only rats with normal motor function and behavior 7 days later were used for experiments. After the study, the location of the catheter was confirmed anatomically and the data of rats with mal-location of the catheter were excluded. In each dose group, eight rats were tested after the data exclusion.

Midazolam (Sigma, St. Louis, MO, USA) and epibatidine (Sigma) were dissolved in normal saline. Epibatidine was adjusted to make solutions of 3, 10, 30, and 100 ng per 10 µl for the formalin test. According to the 50 % effective dose (ED50) in the tail-flick test in previous studies (1.60 µg for midazolam [2] and 32.0 ng for epibatidine [9]), midazolam 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 µg per 5 µl, and epibatidine 2, 4, 8, 16, 32, 64, and 128 ng per 5 µl solutions were made to adjust to 1/16, 1/8, 1/4, 1/2, 1, 2, and 4 times the ED50 dose, respectively. The ED50s for midazolam in the formalin test in a previous study were 1.26 µg in phase 1 and 1.20 µg in phase 2 [2], and those for epibatidine in the present study were 38.0 ng in phase 1 and 27.1 ng in phase 2. From these results, midazolam 0.075, 0.15, 0.3, and 0.6 µg, and epibatidine 1.7, 3.4, 6.8, and 13.6 ng per 5 µl solutions were made to adjust to 1/16, 1/8, 1/4, and 1/2 the ED50 dose, respectively. Equivalent ED50 doses of midazolam and epibatidine were combined for the combination study. Normal saline was used as the control. After injection of the drug, the catheter was flushed with normal saline 10 µl to clear the dead space of the catheter.

The tail-flick test was performed with the Tail-Flick Analgesia Meter (MK-330A; Muromachi Kikai, Tokyo, Japan). Rats were placed in a clear plastic cage with their tails extending through a slot located at the rear of the cage. Thermal stimulation was given by a beam of high-intensity light focused on the tail 2 to 3 cm proximal to the end. The time between the start of the stimulation and tail withdrawal response was measured as the tail-flick latency. The cutoff time in the absence of a response was set at 14 s to prevent tissue injury of the tail. The test was done at 5, 10, 15, 30, 60, 90, 120, 180, and 240 min after drug injection. The data were expressed as the percentage of the maximum possible effect (% MPE):

% MPE = (post-drug latency − pre-drug latency at time 0) × 100 / (cutoff time (14 s) − pre-drug latency at time 0).

The formalin test was performed 10 min after intrathecal drug injection. Fifty microliters of 5% formalin was injected subcutaneously into the dorsal surface of the right hind paw with a 30-G needle. Immediately after the injection, the rat was placed in an open clear plastic chamber and flinching or shaking paw response was observed for 60 min. The number of flinches was counted for 1 min. Usually two phases were observed: phase 1, during 0 to 6 min after formalin injection; and phase 2, beginning about 10 min after the injection, with an interval of no flinches between the two phases.

Side effects in rats were examined and judged as present or absent for the tail-flick test. Agitation was judged as spontaneous irritable movement, vocalization, or both. Allodynia-like behavior was judged as escape, vocalization, or both, induced by lightly stroking the flank of the rat with a small probe. The placing or stepping reflex was evoked by drawing the dorsum of either hind paw across the edge of the table. Normal rats try to put the paw ahead into a position to walk. The righting reflex was assessed by placing the rat horizontally with its back on the table. Normally rats twist the body to an upright position immediately. Flaccidity was judged as muscle weakness when an animal placed the forepaw 3 to 5 cm higher than the hind paw. Normal rats will walk up. The pinna or corneal reflex was examined with a paper string. When a string is put into the ear canal or touches the cornea, rats normally shake their heads.

First, the formalin test was performed with intrathecal epibatidine and the ED50 was calculated. To investigate the interaction between midazolam and epibatidine, an isobolographic analysis was performed by a method based on that of Tallarida et al. [11]. The combinations of 1/4 ED50, 1/2 ED50, ED50, 2ED50, and 4ED50 doses of midazolam and epibatidine in the tail-flick test, and the combinations of 1/16 ED50, 1/8 ED50, 1/4 ED50, and 1/2 ED50 doses of midazolam and epibatidine in the formalin test were tested and the ED50 of the combination was determined. Behavioral side effects were checked simultaneously with the tail-flick test. The ED50 was obtained using the maximum effects in the tail-flick test and the area under the curve of the number of flinches in the formalin test. A total fractional dose value was calculated to describe the magnitude of the interaction, as follows: (ED50 dose of midazolam in combination) / (ED50 dose of midazolam alone) + (ED50 dose of epibatidine in combination) / (ED50 dose of epibatidine alone). The value was normalized by assigning the ED50 value of each drug given alone as 1. Values near 1 suggest an additive interaction,

values of more than 1 imply an antagonistic interaction, and values of less than 1 indicate a synergistic interaction.

The data are shown as means ± SD or 95% confidence intervals (CIs). Statistical analysis to compare the calculated ED50 values with the theoretical additive values was performed with Student's *t* test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

In the tail-flick test, doses of up to 1/2 ED50 induced less than 50% effects; therefore, the combinations of each ED50, 2ED50, and 4ED50 were added together (Fig. 1). The ED50 values of the combination were significantly higher than the theoretical additive values (calculated from the ED50s of midazolam and epibatidine in our previous studies [2,9]; Fig. 2, Table 1). The mean total fractional dose value was 3.19.

In the formalin test, epibatidine showed dose-dependent decreases in the number of flinches in both phase 1 and 2. With doses up to the combination of each 1/2ED50, we could not get ED50 values of the combination; therefore, the combinations of each ED50 were added together. The ED50 values of the combination were significantly lower than the theoretical additive values (calculated from the ED50s of midazolam and epibatidine in our previous studies [2,9]) in phase 1, but they were not significantly different from the theoretical additive values in phase 2 (Fig. 3, 4, Table 1). The mean total fractional dose value was 0.204 for phase 1 and 1.29 for phase 2.

The agitation, allodynia, loss of pinna reflex, and flaccidity seen with each single agent in our previous studies [2,9] were not observed with the combinations administered in the present study. Disturbance of the righting reflex was observed in one rat administered the combination of 4ED50. Disturbance of placing and stepping was observed in one rat administered the combination of 2ED50 and one rat administered with 4ED50.

Discussion

In the present study, the combination of midazolam, a benzodiazepine-$GABA_A$ receptor agonist, and epibatidine, a nicotinic cholinergic receptor agonist, when administered intrathecally, had antagonistic effects on acute thermal nociception, while the combination had synergistic effects on acute inflammatory nociception and additive effects on inflammatory-facilitated nociception,

In the present combination study, we examined for the occurrence of motor disturbance, in consideration

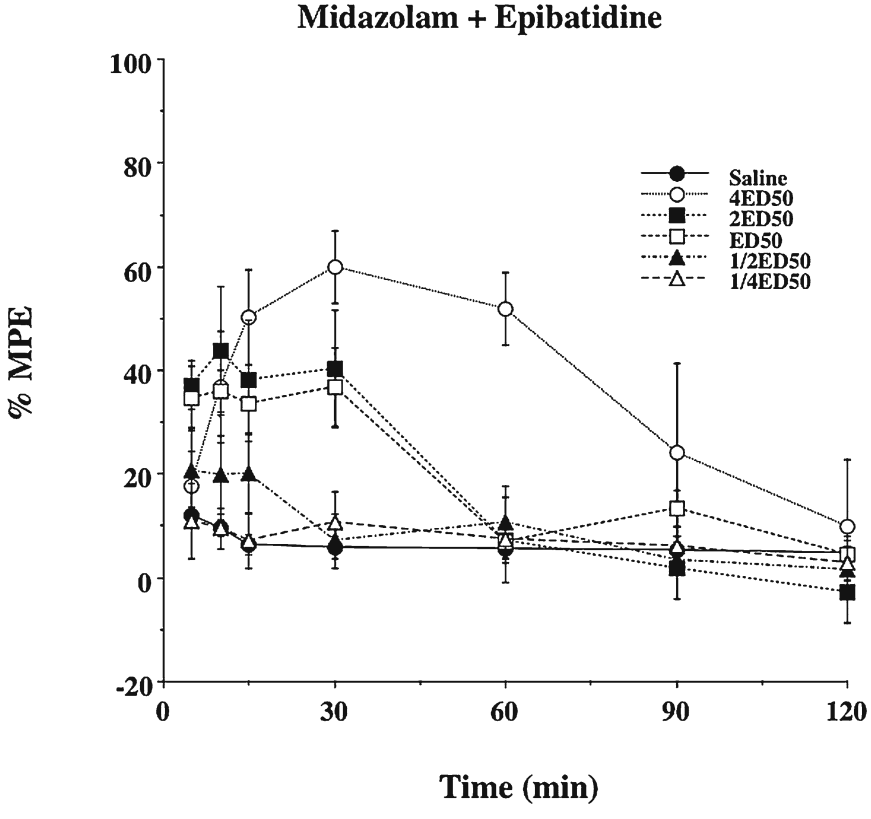

Fig. 1. Time response curves of the effects of intrathecal administration of the combination of midazolam and epibatidine in the tail-flick test. Values are means, and *bars* indicate standard error (n = 8). *% MPF*, percentage of the maximum possible effect; *ED50*, 50% effective dose

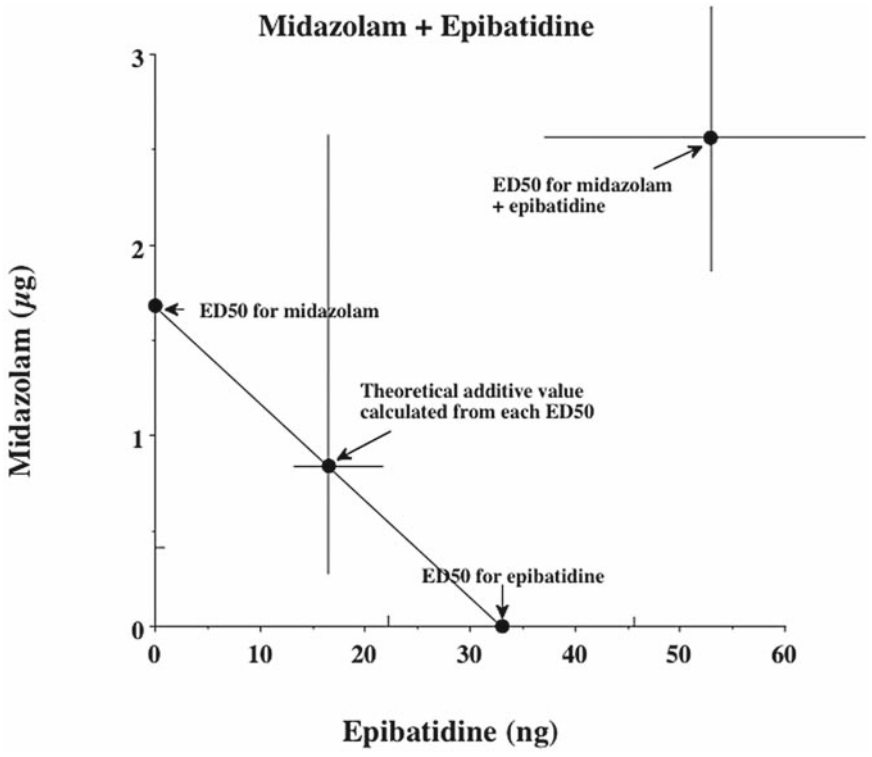

Fig. 2. Isobolograph for the interaction of midazolam and epibatidine in the tail-flick test. *Bars* indicate 95% confidence intervals

Table 1. 50% Effective dose (ED50) values

	Tail-flick test	Formalin test phase 1	Formalin test phase 2
Midazolam (μg)	1.60[a] (0.45–3.02)	1.26[a] (0.35–3.18)	1.20[a] (0.29–3.71)
Epibatidine (ng)	32.0[b] (22.0–46.5)	38.0 (21.5–65.1)	27.1 (10.4–43.5)
Combination (μg) (Midazolam)	2.56* (1.72–3.82)	0.16* (0.05–0.56)	0.84 (0.31–2.25)
Combination (ng) (Epibatidine)	52.9* (35.5–78.9)	3.34* (0.96–11.64)	17.4 (6.4–47.0)

* $P < 0.05$ vs the theoretical additive value (1/2 ED50 of each single agent)
Values in parentheses are 95% confidence intervals
[a,b]The data for midazolam alone and epibatidine alone were derived from previous studies ([a]reference 2; [b]reference 9)

of the results seen with each single agent in our previous studies [2,9]. However, the allodynia and/or agitation, seen in 1/8 rats that received midazolam 10 μg and in 3/8 rats that received epibatidine 100 ng in our previous studies [2,9] were not observed in the present study with the combination of midazolam 6.4 μg and epibatidine 128 ng. The decrease of allodynia and agitation suggests that epibatidine and midazolam may have antagonistic effects in the brain, although the total number of animals tested was too small to show significance.

The GABAergic system plays an important role in the presynaptic inhibition of primary afferents. Midazolam reduced excitatory synaptic transmission by acting on the $GABA_A$/benzodiazepine receptor in interneurons, leading to a decrease in the excitability of spinal dorsal horn neurons [12]. Primary afferent depolarization by $GABA_A$ receptor activation may explain its action on primary afferent terminals of large myelinated A α/β fibers in the deep dorsal horn [13]. Midazolam induced no significant effect on C-fiber-evoked responses, but A δ fiber-evoked responses were markedly and dose-dependently depressed [14].

Nicotine-induced antinociception is dependent upon intact cholinergic neurotransmission in the rostral ventral medulla, which, in turn, activates descending and intrinsic mechanisms in the lumbar spinal cord that modify nociceptive input [15]. Nicotinic binding sites were found mostly in laminae II–III of the dorsal horn, lamina IX (motor neuron area), and lamina X around the central canal of the spinal cord [16]. Immunohistochemical evidence has demonstrated that acetylcholine coexists with GABA in these neurons [17,18]. Therefore, midazolam and the nicotinic acetylcholine receptor agonist, epibatidine, may have some interaction in the spinal cord.

Epibatidine was efficacious in the tonic phase of the formalin test applied to the rat hindpaw [19]. The effects of epibatidine peaked at 10 min post-injection and were

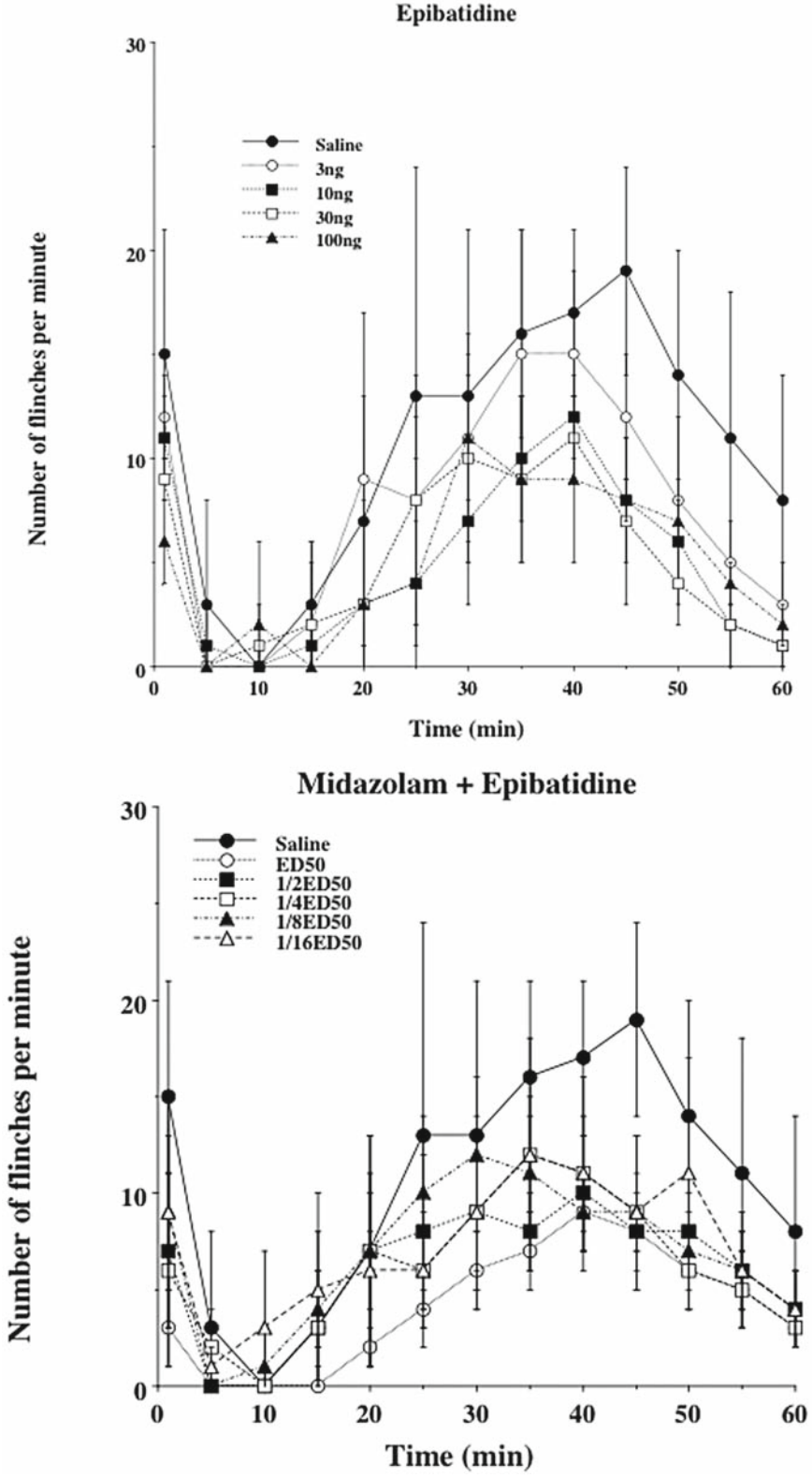

Fig. 3. Time response curves of the effects of intrathecal epibatidine (*upper*), and the combination of midazolam and epibatidine (*lower*) in the formalin test. Values are means, and *bars* indicate standard error ($n = 8$)

completely absent by 30 min [20], while our results showed a longer duration of the effects of intrathecal epibatidine in the tail-flick test [9] and in the formalin test. Epibatidine is approximately 200 to 300 times more potent than nicotine given by various routes [21–23]. However, epibatidine is also extremely toxic, causing hypertension, respiratory paralysis, and seizures, with death occurring at doses not much higher than those

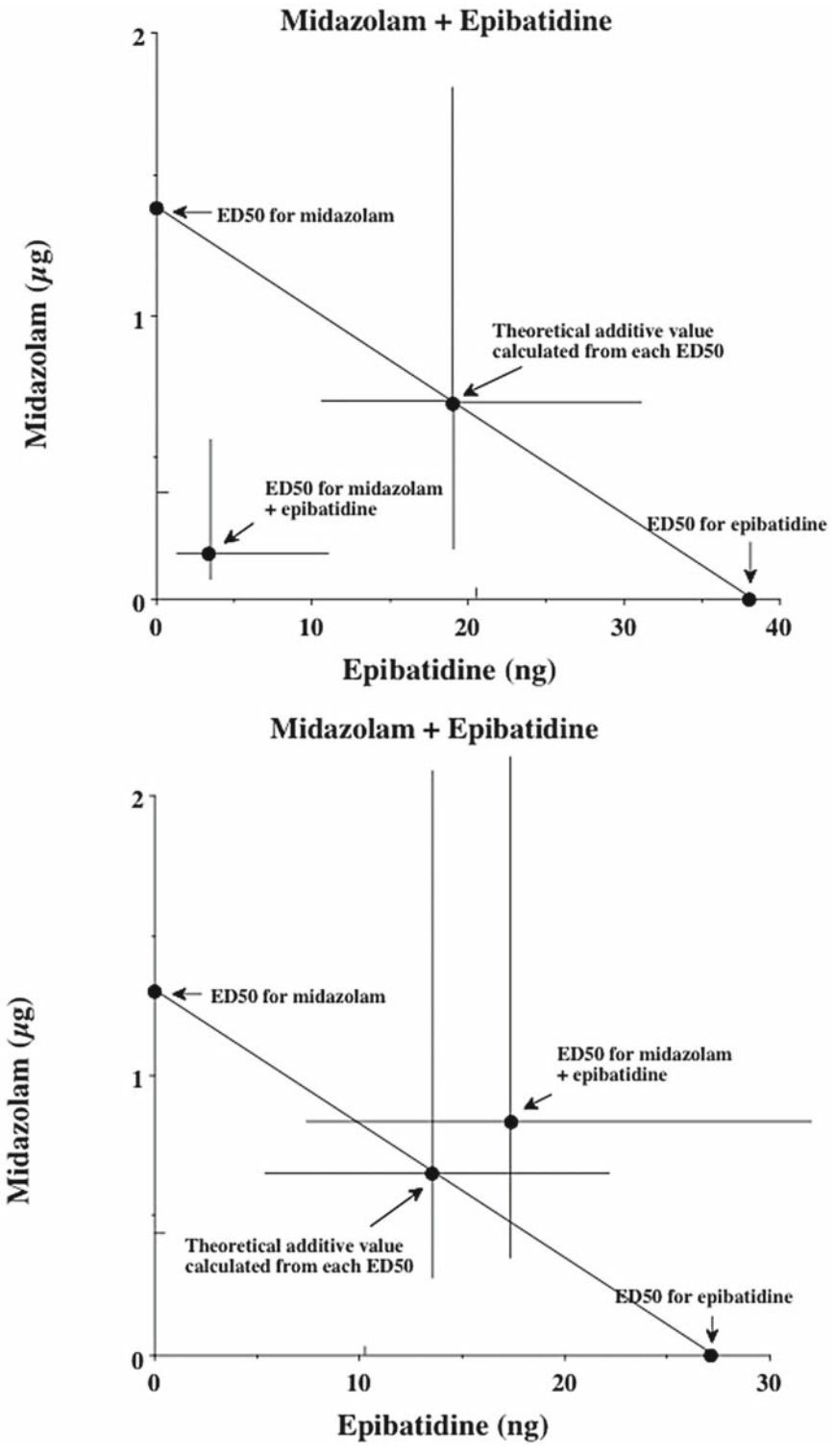

Fig. 4. Isobolographs for the interaction of midazolam and epibatidine in phase 1 (*upper*) and phase 2 (*lower*) of the formalin test. *Bars* indicate 95% confidence intervals

required for antinociception by systemic administration [10,24]. In the present study, we did not find any respiratory paralysis, seizures, or death, although motor disturbance and excitatory behavior (agitation and allodynia) were observed with the intrathecal administration of epibatidine in our previous study [9].

The tail-flick response represents acute thermal nociception that is mediated by primary afferent fibers (A, δ, and C) and in part by spinal reflex [25]. The present results showed antagonistic effects between intrathecal midazolam and epibatidine in the tail-flick test. In rats, intrathecal epibatidine affords a nociceptive response,

which may be caused by the release of excitatory amino acids, at lower doses than its antinociceptive effect [26]. In our previous study [9], some rats administered lower doses of epibatidine showed shortened tail-flick latency, but in total no clear antagonistic effects of epibatidine were seen. Therefore, it is possible that the low doses of epibatidine used in the tail-flick test in the present study may have induced a nociceptive response, which resulted in the antagonistic effects seen with midazolam.

Formalin directly activates peripheral nociceptors on primary afferent fibers, producing an acute barrage of activity into the dorsal horn, which constitutes phase 1 of the response [27]. This reflects activity that is prominent in A β, A δ, and high-threshold C nociceptor afferent fibers. The phase 2 response, which is caused by subsequent inflammation after formalin injection and central sensitization related to C-fiber activity [28], reflects activity in mechanically insensitive afferent fibers and the activity of A δ and C fibers [27].

The synergistic effects of intrathecal midazolam and epibatidine seen in phase 1 of the formalin test in the present study may have been due to an interaction between depressed afferent fiber activity and cholinergic descending inhibition at the spinal cord level. The nociceptive effects of epibatidine seen in thermally induced nociception may not have been activated in inflammatory-induced nociception with the doses used. These different effects might be due to the different sensitivities of nerves related to epibatidine. However, the reason that only additive effects were observed in phase 2 is not known; because usually the phase 2 response is considered to be induced by the phase 1 response.

Another possible reason for the different interactions of midazolam and epibatidine in the tail-flick test, and phases 1 and 2 of the formalin test could be different time courses of uptake, redistribution, and duration of action between intrathecal midazolam and epibatidine. However, from the results of our previous studies [2,9] and the present study, the time courses of the effects of intrathecal midazolam and epibatidine were not so different. Therefore, these interactions may be due to some pharmacological effects rather than different time courses.

In clinical application, intrathecal coadministration of midazolam and epibatidine may be useful for inflammatory pain, although further studies of side effects, including neurotoxicity, are necessary. In contrast, however, intrathecal coadministration of midazolam and epibatidine should not be used for acute thermal pain.

In conclusion, the intrathecal combination of midazolam and epibatidine produced antagonistic effects on acute thermal nociception, while the combination had synergistic effects on acute inflammatory nociception and only additive effects on inflammatory-facilitated nociception.

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