

## ROLE OF NITRIC OXIDE IN BENZODIAZEPINES-INDUCED ANTINOCICEPTION IN MICE

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The influence of nitric oxide (NO) on antinociceptive activity of diazepam (DZ), chlordiazepoxide (CDP) and clonazepam (CZ) was examined using the writhing test in mice. The effect of DZ was also studied in mice using hot plate and tail flick tests. DZ (1.25, 2.5 and 5 mg/kg), CDP (1.25, 2.5, 5, 10 and 20 mg/kg) and CZ (0.075, 0.3125, 0.625, 1.25 and 2.5 mg/kg) produced significant, dose-dependent (DZ, CDP) antinociception in mice. The benzodiazepines (BZs)-induced antinociception was antagonized by flumazenil (5 mg/kg) and was not changed by naloxone (2.5, 5 and 10 mg/kg), except that of CZ, which was reversed by 5 mg/kg of naloxone. N<sup>G</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME) as well as 7-nitroindazole (7-NI) intensified antinociceptive activity of BZs. The antinociceptive effect resulting from co-administration of L-NAME with CZ and 7-NI with CDP was reversed by L-arginine. Methylene blue (MB) increased, whereas L-arginine (but not D-arginine) decreased antinociceptive effects of the studied BZs. These results suggest that the NO-cGMP pathway is involved in the mechanism of BZs-induced antinociception in the writhing test in mice.

**Key words:** nitric oxide, benzodiazepines, nociception, mice

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## INTRODUCTION

The benzodiazepines (BZs) are generally known as sedative, antianxiety, hypnotic and anti-convulsant drugs. They exert their pharmacological effects *via* interaction with specific recognition sites that are part of the macromolecular GABA receptor complex. BZs bind to  $\gamma$ -aminobutyric acid-A (GABA<sub>A</sub>) receptors and act to enhance the action of the inhibitory neurotransmitter GABA at these receptors [13]. Several observations indicate an antinociceptive effect of BZs [12, 32] while other findings do not support those observations [25, 28, 29]. Despite these discrepancies in research data, the BZs have gained widespread clinical acceptance as adjuvants in the management of several specific painful states. They are recommended for pain associated with anxiety, pain due to muscle injury and spasm, and neuropathic pain [26].

Nitric oxide (NO) is a free-radical gas with strong biological properties formed by the enzyme NO synthase from L-arginine. NO is known to activate soluble guanylate cyclase and to increase the intracellular content of cGMP, which may modulate a great number of physiological functions [33]. There are evidences that NO may be involved both peripheral and central nociceptive processing [5, 8, 16, 34]. The number of *in vivo* and *in vitro* studies suggest that NO plays a modulatory role in either release or uptake of neurotransmitters including glutamate [11] and GABA [11, 19, 30]. Histochemical mapping of NO synthase revealed that NO synthase-positive neurons are co-localized with GABA [36]. It has also been postulated that NO can modulate the activity of GABA<sub>A</sub> receptors [39] or act directly on GABA<sub>A</sub> receptors [18].

The aim of this paper was to estimate the role of NO:cGMP pathway in antinociceptive effects of BZs, the activity of which is closely connected with GABAergic neurotransmission.

## MATERIALS and METHODS

### Animals

The experiments were carried out on male Albino Swiss mice (18–26 g). The animals were kept 8–10 to a cage under standard laboratory conditions (at a temperature of  $20 \pm 1^\circ\text{C}$  and a 12 h light/dark cycle) with free access to food and water. All experiments were performed between 9:00 a.m. and 4:00 p.m.

The experiments were performed in accordance with the ethical requirements.

### Drugs

The following drugs were used: diazepam (DZ, Relanium, Polfa, Poland), chlordiazepoxide hydrochloride (CDP, Elenium, Polfa, Poland), clonazepam (CZ, Rivotril, Hoffmann-La Roche, Germany), N<sup>G</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma, USA), 7-nitroindazole (7-NI, RBI, USA), flumazenil (Hoffmann-La Roche, Germany), and methylene blue (MB), L-arginine hydrochloride, D-arginine hydrochloride, and naloxone hydrochloride (all from Sigma, USA).

### Procedure

The following tests were used to investigate the nociceptive reaction in mice.

1. The writhing test [15]. The number of writhing episodes was counted during a 10 min period, starting 5 min after intraperitoneal (*ip*) administration of 0.6% acetic acid solution in a volume of 10 ml/kg.

MB was administrated intravenously (*iv*) at the dose of 5 mg/kg 5 min before the acid. Other substances were administered *sc*: the BZs, L-NAME, 7-NI and naloxone 30 min before the acid, flumazenil 15 min prior to acid administration, L- and D-arginine 35 min prior to acid administration. Treatments that produced a significant decrease in the number of writhing episodes compared to that of vehicle-injected control mice tested on the same day were considered to be antinociceptive. The absolute mean values of writhing episodes in control group ranged from  $22.5 \pm 1.0$  to  $32.0 \pm 2.8$  ( $\pm$  SEM).

2. The hot plate test [6]. The animals were placed at constant temperature of  $56^\circ\text{C}$  and the latency of pain response (paw licking, jump) was measured.

3. The tail flick test [3]. The animal's tail was placed in a hot water bath at a temperature of  $52^\circ\text{C}$  and the latency of response (rapid tail movement) was measured.

In both tests the cutoff time was 20 s. Each animal was tested 15 min before and 15, 30, 60 and 90 min after DZ (1.25, 2.5 and 5 mg/kg *sc*) administration.

### Statistical analysis

Data are expressed as the means ( $\pm$  SEM). Statistical significance of differences between groups was determined by Student's *t*-test.

RESULTS

The antinociceptive effects of benzodiazepines in the writhing test in mice

DZ (1.25, 2.5 and 5 mg/kg), CDP (1.25, 2.5, 5, 10 and 20 mg/kg) and CZ (0.075, 0.3125, 0.625, 1.25 and 2.5 mg/kg) produced significant, dose-dependent (DZ and CDP but not CZ) decrease in the number of writhing episodes in mice (Fig. 1).

The influence of flumazenil and naloxone on antinociceptive effects of benzodiazepines in the writhing test in mice

Flumazenil (5 mg/kg) was able to antagonize antinociceptive effects of DZ (2.5 mg/kg), CDP (5 mg/kg) and CZ (2.5 mg/kg). The BZs-induced antinociception was nonsignificantly attenuated by naloxone (2.5, 5 and 10 mg/kg),

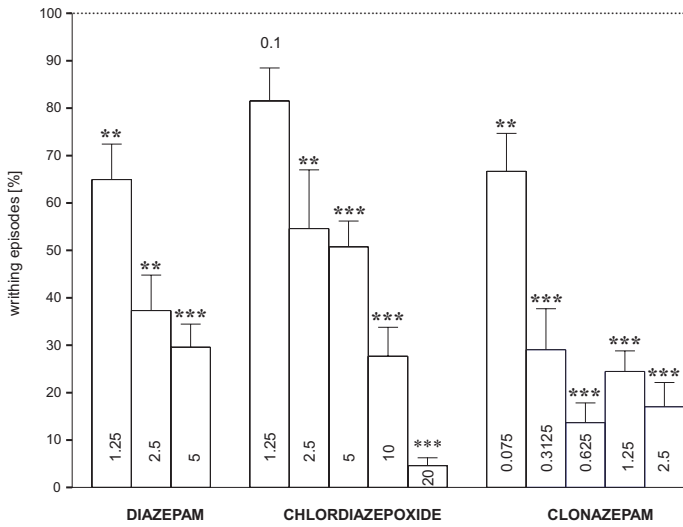


Fig. 1. The antinociceptive effects of diazepam, chlordiazepoxide and clonazepam in the writhing test in mice. The results are expressed as means ± SEM of groups consisting of 8 (10) mice. The mean value of a number of writhing episodes in the respective control group was assumed to be 100%. \*\* p < 0.01, \*\*\* p < 0.001 vs control

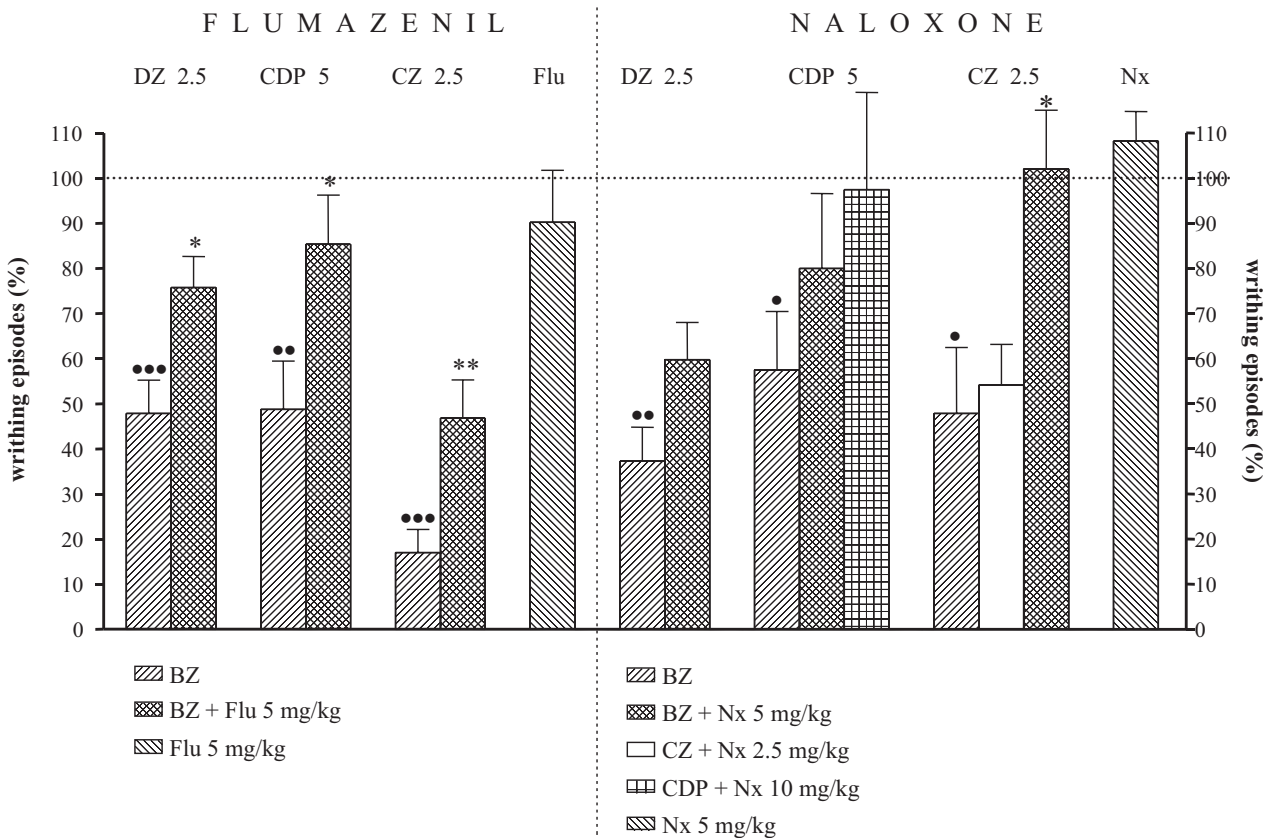


Fig. 2. The influence of flumazenil (Flu) and naloxone (Nx) on antinociceptive effects of diazepam (DZ), chlordiazepoxide (CDP) and clonazepam (CZ) in the writhing test in mice. The results are expressed as means ± SEM of groups consisting of 8 (10) mice. The mean value of a number of writhing episodes in the respective control group was assumed to be 100%. • p < 0.05, •• p < 0.01, ••• p < 0.001 vs control; \* p < 0.05, \*\* p < 0.01 vs benzodiazepine (BZ)

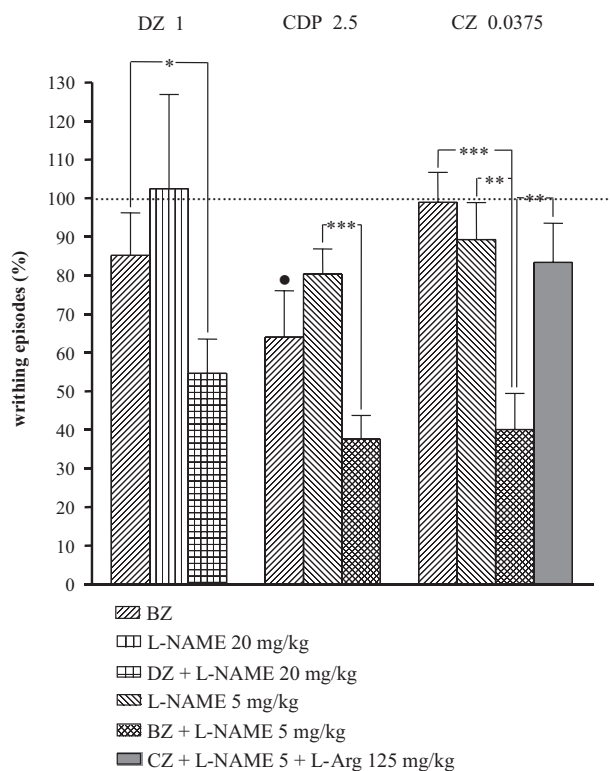


Fig. 3. The influence of  $N^G$ -nitro-L-arginine methyl ester (L-NAME) on antinociceptive effects of diazepam (DZ), chlordiazepoxide (CDP) and clonazepam (CZ) in the writhing test in mice. The results are expressed as means  $\pm$  SEM of groups consisting of 8 (10) mice. The mean value of a number of writhing episodes in the respective control group was assumed to be 100%. \*  $p < 0.05$  vs control; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

except CZ activity, which was reversed by 5 mg/kg of naloxone (Fig. 2).

### The influence of L-NAME on antinociceptive effects of benzodiazepines in the writhing test in mice

Co-administration of L-NAME (20 or 5 mg/kg) with DZ and CZ (at noneffective doses when given alone) or CDP resulted in significant antinociception. L-arginine (125 mg/kg) was able to reverse the effects produced by co-administration of L-NAME (5 mg/kg) with CZ (0.0375 mg/kg) (Fig. 3).

### The influence of 7-nitroindazole on antinociceptive effects of benzodiazepines in the writhing test in mice

Co-administration of 7-NI (0.5 mg/kg) with DZ, CDP and CZ (at the threshold dose) resulted in

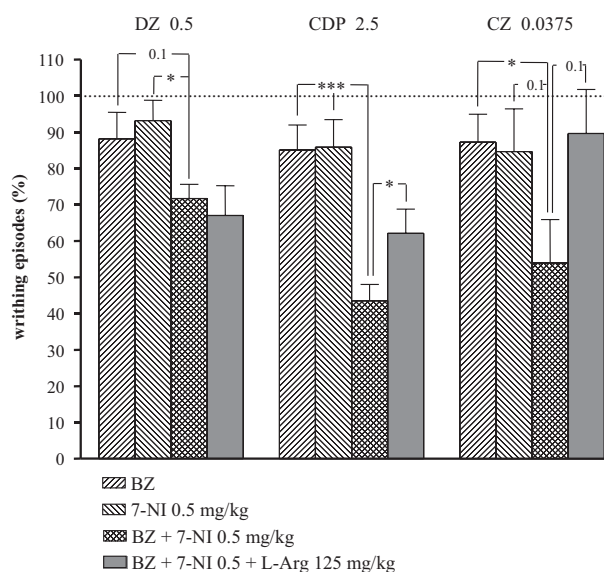


Fig. 4. The influence of 7-nitroindazole (7-NI) on antinociceptive effects of diazepam (DZ), chlordiazepoxide (CDP) and clonazepam (CZ) in the writhing test in mice. The results are expressed as means  $\pm$  SEM of groups consisting of 8 (10) mice. The mean value of a number of writhing episodes in the respective control group was assumed to be 100%. \*  $p < 0.05$ , \*\*\*  $p < 0.001$

significant and remarkable antinociception. L-arginine (125 mg/kg) was able to attenuate the effects produced by co-administration of CDP with 7-NI and CZ with 7-NI but not that of DZ with 7-NI (Fig. 4).

### The influence of methylene blue on antinociceptive effects of benzodiazepines in the writhing test in mice

The antinociceptive action of the threshold doses of DZ or CZ was intensified by MB (5 mg/kg *iv*) as shown in Figure 5.

### The influence of L-arginine and D-arginine on antinociceptive effects of benzodiazepines in the writhing test in mice

Antinociceptive effects of DZ (2.5 mg/kg) were significantly reduced by L-arginine (125 mg/kg). L-arginine (125 mg/kg) but not D-arginine (125 mg/kg), was able to attenuate the antinociceptive activity of CZ (2.5 mg/kg) (Fig. 6).

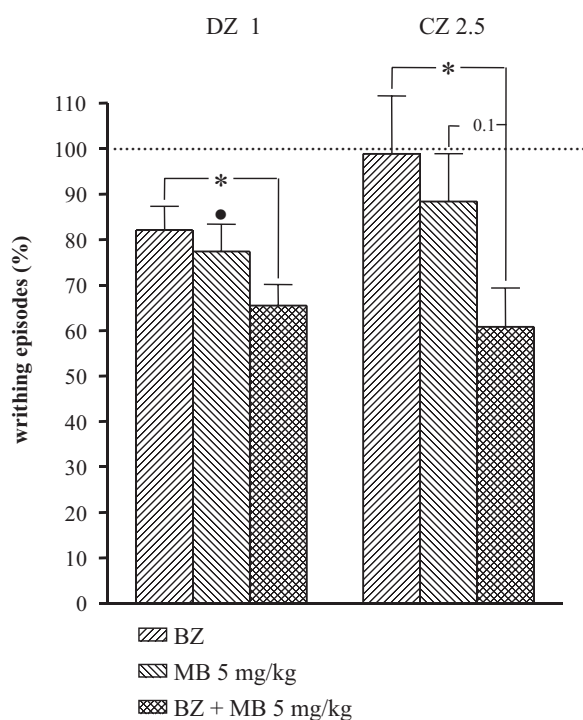


Fig. 5. The influence of methylene blue (MB) on antinociceptive effects of diazepam (DZ) and clonazepam (CZ) in the writhing test in mice. The results are expressed as means  $\pm$  SEM of groups consisting of 8 (10) mice. The mean value of a number of writhing episodes in the respective control group was assumed to be 100%. \*  $p < 0.05$  vs control; \*  $p < 0.01$

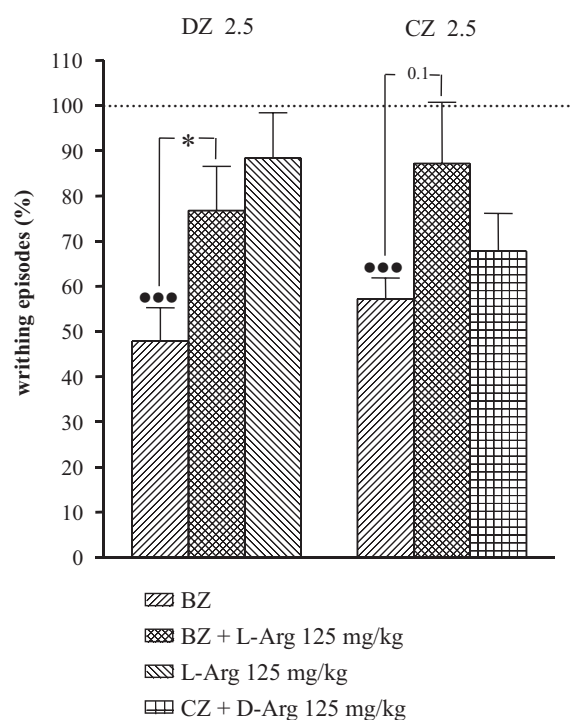


Fig. 6. The influence of L-arginine (L-Arg) and D-arginine (D-Arg) on antinociceptive effects of diazepam (DZ) and clonazepam (CZ) in the writhing test in mice. The results are expressed as means  $\pm$  SEM of groups consisting of 8 (10) mice. The mean value of a number of writhing episodes in the respective control group was assumed to be 100%. \*\*\*  $p < 0.001$  vs control; \*  $p < 0.01$

## DISCUSSION

BZs are very useful drugs in the treatment of anxiety, insomnia and epilepsy. The anxiolytic and sedating effects of BZs have led to their widespread use as adjuncts to anesthetics during surgical procedures. It has also been documented that BZs are widely used in the management of chronic pain [14]. The antinociceptive action of BZs has been studied in different animal models, but the conclusions are contradictory. A direct antinociceptive effect of BZs has been demonstrated using the hot plate test [7], tail-flick test [38] and writhing test [32]. Furthermore, BZs have been shown to potentiate opioid antinociception in animals [25].

There is evidence that L-arginine: nitric oxide: cGMP pathway may be involved in peripheral and central nociceptive processing. It has been reported that increased NO production induces hyperalgesia and that NOS-inhibitors can suppress pain [22]. Paradoxically, results from recent research have also implicated NO as a mediator or modulator in

analgesic drug function. Among drug effects that apparently involve an L-arginine : NO : cGMP pathway, the peripheral analgesic action of morphine [5], the central analgesic mechanisms of  $\beta$ -endorphin [35] should also be mentioned.

A great body of evidence points to interactions between NO and GABA. For example, high concentration of NO have been shown to act presynaptically to potentiate the release of GABA [10, 11, 19] and postsynaptically to potentiate GABA-mediated increases in chloride conductance at GABA<sub>A</sub> receptors [9]. In contrast, low concentrations of NO can exert inhibitory effects on GABAergic transmission [9, 10] indicating that the effects of NO can be biphasic.

In the present studies, we have shown that BZs were able to induce antinociceptive action in mice in the writhing test. In this test both central and peripheral analgesics could be evaluated, and many investigators use it and recommend as a simple screening method [37]. In addition, the writhing test possesses high sensitivity and it makes possible

to detect analgesic activity of non-steroidal anti-inflammatory drugs. Narcotic analgesics (e.g. morphine) are effective at ten times lower doses in the writhing test than doses effective in the hot plate or tail flick test [data not shown]. The antinociceptive action of BZs in this test is not due to a spasmolytic effect of BZs, since this property has not been described for this type of drugs [2] and also it was not antagonized by atropine at 0.1–1 mg/kg [32]. The described effect is dose-dependent and is related to BZ receptor, since antinociceptive action of BZs was antagonized by flumazenil. Moreover, this antinociceptive effect of BZs seems not to be related to opioid receptors since it was slightly changed by naloxone, except perhaps for CZ activity, which was reversed by 5 mg/kg of naloxone. However, it is generally known that this dose of naloxone may affect not only the opioid receptors.

The antinociceptive effects of BZs were also studied in thermal tests, i.e. hot plate and tail flick tests. The observed activities were slight, only incidentally significant and not dose-dependent [data not shown]. Therefore the writhing test was chosen to further studies.

It is known that competitive inhibition of NO synthase by L-NAME [27] or 7-NI [21] leads to the reduction of the biosynthesis of NO from L-arginine. It has also been found that NO synthase inhibitors attenuated behavioral pain responses when applied parenterally [23, 24], topically to the spinal cord [22] and also *icv* [23]. A variety of NOS inhibitors have been used to explore the role of NO in pain transmission. L-NAME is a nonselective antagonist that inhibits the activity of both endothelial and neuronal NO synthase and causes pronounced increases in arterial blood pressure [27], while 7-NI has been described as a selective inhibitor of neuronal NO synthase *in vivo* [1]. Although neither L-NAME nor 7-NI altered responses in the tail flick assay when thermal or mechanical stimulation were used [31], both compounds had antinociceptive effects in mice when tested using the writhing assay [8, 16, 17] or the formalin test [23, 24]. The results of the present study demonstrate that *sc* administration of L-NAME as well as 7-NI intensified antinociceptive activity of BZs. L-arginine, endogenous donor of NO, reversed the effect resulting from co-administration of L-NAME with CZ and 7-NI with CDP.

MB has been widely applied in experiments as a simple inhibitor of guanylate cyclase [22]. Since

guanylate cyclase is one of the main targets of NO [4], MB is often used to determine the contribution of the cGMP pathway in the effects of NOergic system. Moreover, Mayer et al. [20] have shown that MB acts *in vitro* as a direct inhibitor of purified cerebellar NO synthase.

We also observed that MB produced antinociception in mice, as it caused a decline in the number of writhing episodes after *ip* injection of acetic acid [data not shown]. Co-administration of MB (at nonanalgesic dose when given alone) with the studied BZs resulted in the increase in their antinociceptive effects.

We also examined the ability of L-arginine, endogenous donor of NO, to reverse the antinociceptive effects of BZs. Administration of L-arginine (but not D-arginine) resulted in the expected decrease in BZs-induced antinociception.

In summary, the antinociceptive activity of BZs expressed as a number of acetic acid-induced writhing episodes in mice is dependent on GABAergic mechanisms and seems to be slightly connected with opioid system. Intensification of BZs-induced antinociception by administration of L-NAME or 7-NI, could be interpreted as resulting from reduction of the level of NO caused by the inhibition of NO synthase. Moreover, increasing of the production of NO by administration of L-arginine, endogenous donor of NO, reversed antinociceptive effect of BZs. The participation of NO : cGMP pathway in the antinociceptive effects of BZs is also supported by intensification of these effects by MB, inhibitor of guanylate cyclase. Additionally, intensification of BZs-induced antinociception by 7-NI, relatively selective inhibitor of neuronal NO synthase, seems to point on central origin observed activities. Because the peripheral origin of antinociceptive effects of BZs cannot be excluded, the further studies are planned to elucidate this problem.

In conclusion, the results of the present study indicate that NO is involved, at least partly, in the antinociceptive activity of BZs.

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