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Effect of nitric oxide synthase inhibitors on benzodiazepine withdrawal in mice and rats

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Abstract:
This study was undertaken to evaluate the effect of nitric oxide (NO) synthase inhibitors on benzodiazepine withdrawal syndrome in mice and rats. Diazepam withdrawal in mice was read out as intensification of the seizures induced by a subthreshold dose of pentetrazole. In rats, the withdrawal syndrome resulting from chronic administration of diazepam, chlordiazepoxide, clonazepam and temazepam was characterized by audiogenic seizures, hypermotility and weight loss. Administration of the non-selective NO synthase inhibitors N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) significantly attenuated the withdrawal syndrome (i.e., pentetrazole-induced seizures) in diazepam-dependent mice. L-NOARG significantly suppressed hypermotility in clonazepam-dependent rats and inhibited the decrease in body weight observed after 12 h of withdrawal in chlordiazepoxide- and clonazepam-dependent rats. Moreover, a clear propensity of L-NOARG to protect benzodiazepine-dependent rats against audiogenic seizures was observed. These findings suggest that the cGMP/NO system may participate in causing the signs of benzodiazepine withdrawal.

Key words:
nitric oxide, benzodiazepines, withdrawal, mice, rats

Introduction

Benzodiazepines are the most commonly prescribed psychoactive drugs and exert a number of pharmacological effects, such as anxiolysis, sedation, hypnosis, muscle relaxation and anticonvulsant activity. It is generally known that the main factors involved in the effects of benzodiazepines are γ-aminobutyric acid (GABA) and the GABAergic system [3, 28, 29, 60]. Unfortunately, chronic benzodiazepine treatment may lead to the development of tolerance and dependence [60]. Although multiple chemical mediators are now hypothesized to be involved in the addictive effect of benzodiazepines, the cellular and neural mechanisms involved in the development and expression of benzodiazepine dependence are not fully understood.

Nitric oxide (NO) is a diffusible second messenger formed from the amino acid L-arginine by the enzyme NO synthase upon activation of NMDA receptors [11]. It has been well established that one of the pathways for NO signaling begins with the activation of guanyl cyclase, resulting in an increase in the level of the intracellular second messenger cGMP [11, 31]. NO appears to be a novel neuronal messenger involved in a number of physiological and pathophysiological processes, e.g., nociception [42], neurogenesis [6], learning and memory [62], anxiety [37] and seizure activity [9]. Recent studies have established that the NO pathway contributes to neuronal adaptation in response to repeated exposure to a variety of addictive drugs [55] and also that the inhibition of NO production attenuates the signs of withdrawal from opioids [10, 57], ethanol [54], psychostimulants [38] and nicotine [39].

Data from the literature indicate that there is a relationship between the L-arginine:NO:cGMP pathway and GABA-mediated synaptic transmission in the central nervous system. A number of *in vivo* and *in vitro* experiments suggest that NO plays a modulatory role in either the release or the uptake of several neurotransmitters in the brain, including GABA [26, 41]. Valtschanoff's group [56] has provided evidence for the co-localization of NO synthase with GABA in neurons of the rat cortex, hippocampus and striatum. It has also been postulated that NO can modulate the activity of $GABA_A$ receptors [63] or act directly on $GABA_A$ receptors [23]. In our previous experiments we showed that NO may be involved in some of the acute effects of benzodiazepines, such as their antinociceptive [48], anticonvulsant [47] and hypnotic activities [46], and also in the development of tolerance after chronic treatment with diazepam [49].

In the light of these findings, there is a need to determine the role of NO in the occurrence of benzodiazepine withdrawal signs in mice and rats. Diazepam ($T_{0.5}$ = 43 ± 13 h) and chlordiazepoxide ($T_{0.5}$ = 10 ± 3 h) were chosen as representatives of the major clinically available long-acting benzodiazepines because of their long-acting metabolite desmethyldiazepam. In contrast, clonazepam ($T_{0.5}$ = 23 ± 3 h) and temazepam ($T_{0.5}$ = 11 ± 6 h) were chosen as representatives of intermediate- and short-acting benzodiazepines, respectively [7]. The influence of L-NAME and L-NOARG on hypersusceptibility to pentetrazole-induced seizures was evaluated in diazepam-withdrawn mice. The effect of L-NOARG on the withdrawal signs resulting from long-lasting exposure to diazepam, chlordiazepoxide, clonazepam and temazepam (audiogenic seizures, hypermotility and body weight loss) was studied in rats. Both L-NAME and L-NOARG are nonselective NO synthase inhibitors. Apart from their central activity, they also affect the cardiovascular system and increase arterial blood pressure [40], but they are widely used in preliminary behavioral studies.

Materials and Methods

Animals

The experiments were carried out on male albino Swiss mice (23–30 g) and male Wistar rats (170–220 g). The animals were housed in groups of 10/cage at room temperature (22 ± 1°C) and in a natural day-night cycle. Standard food and water were available *ad libitum* over the whole period of the experiments. All experiments were performed between 9:00 a.m. and 3:00 p.m.

All behavioral experiments were carried out according to both the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the European Community Council Directive for the Care and Use of Laboratory Animals of 24 November 1986 (86/609/EEC) and were approved by the local ethics committee.

Drugs

The following drugs were used: diazepam (Relanium, Polfa, Poland), chlordiazepoxide, clonazepam, temazepam (all from Polfa, Poland), N^G-nitro-L-arginine (L-NOARG, RBI, USA), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma-Aldrich, USA), flumazenil (RO-151788, Hoffman-La Roche, Switzerland) and pentetrazole (Sigma-Aldrich, USA). The benzodiazepine pellets were prepared according to the modified procedure described by Way et al. [59] for morphine pellets.

L-NOARG, L-NAME and pentetrazole were dissolved in saline. Diazepam was diluted in saline to the proper concentration. Flumazenil was dissolved in the minimal volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and diluted in saline. All benzodiazepines and pentetrazole were administered subcutaneously (*sc*), and all other drugs were injected intraperitoneally (*ip*). The drugs were administered in a volume of 10 ml/kg for mice and 5 ml/kg for rats. The control animals were injected with an appropriate volume of the solvent.

Experimental procedure

Diazepam dependence was induced in mice by subcutaneous (*sc*) implantation of 1 pellet containing 75 mg/kg of DZ, which was left implanted for 14 days. Additionally, the animals were injected *sc* with diazepam at a dose of 50 mg/kg/day for 11 days (from the 4th to 15th day of pellet implantation). On the 15th day, the pellets were removed, and an hour later, the last dose of diazepam was administered. The experiments were conducted 48 h after removal of the pellets.

Dependence on diazepam, chlordiazepoxide, clonazepam and temazepam was obtained in rats by *sc* implantation of 2 pellets containing 75 mg/kg of each

benzodiazepine; the pellets were left in for 21 days and removed on the 22nd day; studies were performed about 10 h after pellet removal.

The control animals were implanted with placebo pellets and received an equal volume of the solvent at the proper time before the test.

The level of withdrawal after the discontinuation of chronic administration of diazepam in mice was estimated by the amount of intensification of the seizures induced by the concomitant administration of a subthreshold dose (55 mg/kg) of pentetrazole and benzodiazepine receptor antagonist – flumazenil (10 mg/kg). The NO synthase inhibitors were injected 10 min before pentetrazole and flumazenil. The animals were placed singly in glass cylinders after pentetrazole administration, and over the next 60 min, the number of mice developing clonic seizures and tonic convulsions, the number of episodes and the number of animals that died was recorded. The absolute mean values of the numbers of clonic episodes and tonic episodes and the mortality rate in the control group (treated with pentetrazole and flumazenil alone) were taken as 100%.

In rats, signs of benzodiazepine withdrawal after the discontinuation of chronic treatment with benzodiazepines were induced by the injection of flumazenil (10 mg/kg) 5 min before the test. To evaluate locomotor activity, rats were placed singly in round actometer cages (32 cm diameter, two light beams) and observed for 30 min. Audiogenic seizures were evoked by the sound of an electric bell (92 dB, lasting up to 60 s). The weight of the rats was recorded every day after the implantation of pellets and 6, 12 and 24 h after removal of the pellets.

L-NAME (50, 100, 200 mg/kg) and L-NOARG (7.5, 75 mg/kg) were injected 30 min before the experiments.

Statistical analysis

The obtained data were evaluated statistically using a χ^2 test with Yates correction (for the number of mice with pentetrazole-induced and audiogenic seizures) and one-way ANOVA with Tukey-Kramer's *post-hoc* test (for the number of seizure episodes in pentetrazole-induced convulsions, locomotor activity, and weight loss). The results are expressed as the means ± SEM of groups consisting of 10 animals. A probability (p) value of 0.05 or less was considered to be statistically significant.

Results

The influence of L-NAME on diazepam withdrawal syndrome (pentetrazole-induced seizures) in mice

In diazepam-treated mice, a strong withdrawal syndrome, manifested by intensification of the seizures evoked by a subthreshold dose of pentetrazole (55 mg/kg), was only observed after the previous administration of flumazenil (10 mg/kg) (Fig. 1A). In total, this treatment resulted in 21 clonic seizure episodes, 4 tonic seizure episodes in mice and 3 deaths. These values were taken as 100% (control group), and the results for all other groups were compared to them. Administration of L-NAME (at 50, 100 and 200 mg/kg) significantly decreased the number of clonic convulsions ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively) and protected the diazepam-dependent mice against tonic seizures ($p < 0.05$). It also resulted in a non-significant protection against mortality. Pretreatment with L-NAME (200 mg/kg) combined with pentetrazole and flumazenil treatment did not affect seizure activity in mice previously implanted with placebo pellets.

The influence of L-NOARG on diazepam withdrawal syndrome (pentetrazole-induced seizures) in mice

In diazepam-treated mice, co-administration of a subthreshold dose of pentetrazole (55 mg/kg) with flumazenil (10 mg/kg) induced a withdrawal syndrome, which manifested as 18 clonic seizure episodes, 4 tonic seizure episodes and 4 deaths overall (Fig. 1B). These values were taken as 100% (control group), and the results for all other groups were compared to them. L-NOARG (7.5 and 75 mg/kg) significantly and fully protected the diazepam-dependent animals against tonic convulsions ($p < 0.05$) and death ($p < 0.05$). Administration of L-NOARG at the higher dose (75 mg/kg) also reduced the number of clonic seizures in mice chronically treated with diazepam ($p < 0.01$). Pretreatment with L-NOARG (75 mg/kg) combined with pentetrazole and flumazenil treatment did not affect seizure activity in mice implanted with placebo pellets.

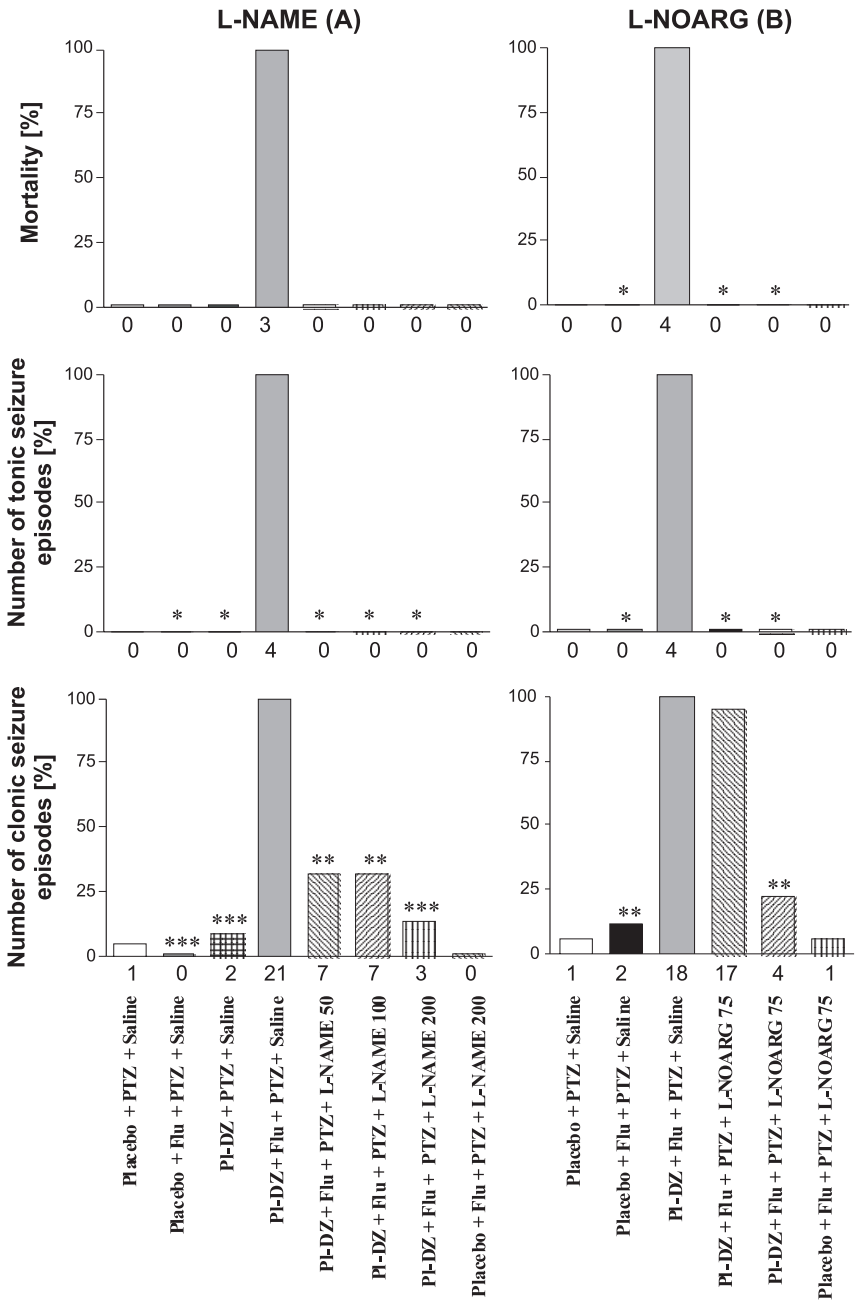

Fig. 1. The influence of **A**. L-NAME (50, 100 and 200 mg/kg) and **B**. L-NOARG (7.5 and 75 mg/kg) on diazepam withdrawal manifested by pentetrazole-induced seizures in mice. The results are expressed as the means ± SEM for the number of seizure episodes and the mortality rate (in %) in n = 10 mice. The mean values of the number of clonic and tonic episodes and the mortality rate in mice treated with diazepam (Pl-DZ) + flumazenil (Flu) + pentetrazole (PTZ) + saline (control mice) were assumed to be 100%. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control (*post-hoc* Tukey-Kramer's test)

The influence of L-NOARG on the hypermotility associated with diazepam, chlordiazepoxide, clonazepam and temazepam withdrawal in rats

Administration of flumazenil (10 mg/kg) in rats chronically treated with diazepam, chlordiazepoxide, clonazepam and temazepam induced strong and significant ($p < 0.05$, $p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively) withdrawal signs, which manifested as an increase in locomotor activity (Fig. 2). L-NOARG (15 mg/kg) significantly reduced the locomotor activity in rats chronically treated with diazepam ($p < 0.05$) and clonazepam ($p < 0.05$) but not those treated with chlordiazepoxide and temazepam. Locomotor activity in benzodiazepine-dependent rats was not altered by the administration of the lower dose of L-NOARG (7.5 mg/kg).

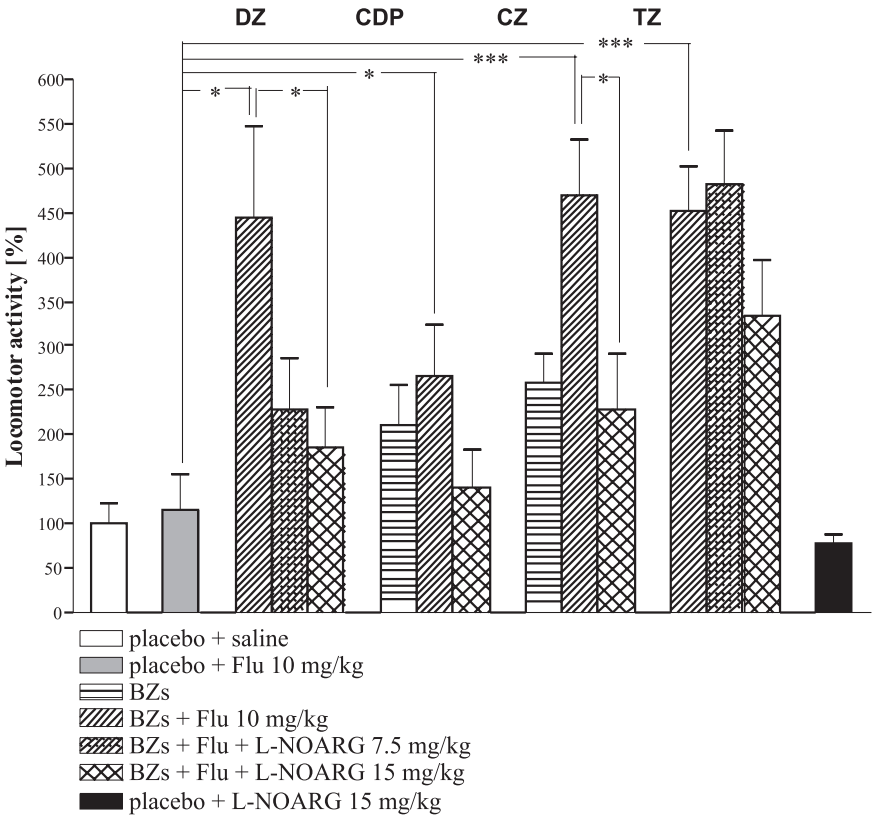

Fig. 2. The influence of L-NOARG (7.5 and 15 mg/kg) on hypermotility during flumazenil (Flu)-induced withdrawal from the following benzodiazepines (BZs): diazepam (DZ), chlordiazepoxide (CDP), clonazepam (CZ) and temazepam (TZ). The results are expressed as the means ± SEM for n = 10 rats. The mean value for the locomotor activity in rats implanted with placebo pellets was assumed to be 100%. * $p < 0.05$, *** $p < 0.001$ (*post-hoc* Tukey-Kramer's test)

Effect of L-NOARG on audiogenic seizures associated with diazepam, chlordiazepoxide, clonazepam and temazepam withdrawal in rats

Discontinuation of benzodiazepine administration followed by an injection of flumazenil (10 mg/kg) and application of a sound of an electric bell (92 dB, lasting up to 60 s) significantly evoked audiogenic seizures in rats treated with chlordiazepoxide ($p < 0.05$) and clonazepam ($p < 0.01$) (Fig. 3). There were no significant differences in the frequency of audiogenic seizures after administration of L-NOARG (7.5 and 15 mg/kg) in benzodiazepine-dependent rats.

Effect of L-NOARG on body weight loss associated with diazepam, chlordiazepoxide, clonazepam and temazepam withdrawal in rats

There were no significant differences in body weight gain between the chronically benzodiazepine-treated rats and the placebo group during the development of benzodiazepine dependence (Fig. 4). Discontinuation of benzodiazepine administration alone (in the case of chlordiazepoxide and clonazepam) or combined with an injection of flumazenil (10 mg/kg) resulted in significant body weight loss in diazepam-treated rats at 6 h ($p < 0.001$) and 12 h ($p < 0.05$), in temazepam-treated rats at 6 h ($p < 0.01$) and in chlordiazepoxide- and clonazepam-treated rats at 12 h after the removal of the pellets. L-NOARG was administered at 7.5 and 15 mg/kg; however, only the higher dose resulted in inhibition of weight loss in chlordiazepoxide- and clonazepam-dependent rats at 12 h after removal of the pellets.

Discussion

Long-term exposure to benzodiazepines has been shown to produce tolerance and dependence in humans and several animal species [20, 60]. In humans, discontinuation of the chronic administration of benzodiazepines induces a withdrawal syndrome, which involves various signs and symptoms including enhanced anxiety, hyperactivity, insomnia, reduced seizure threshold and perceptual disturbances [60]. In

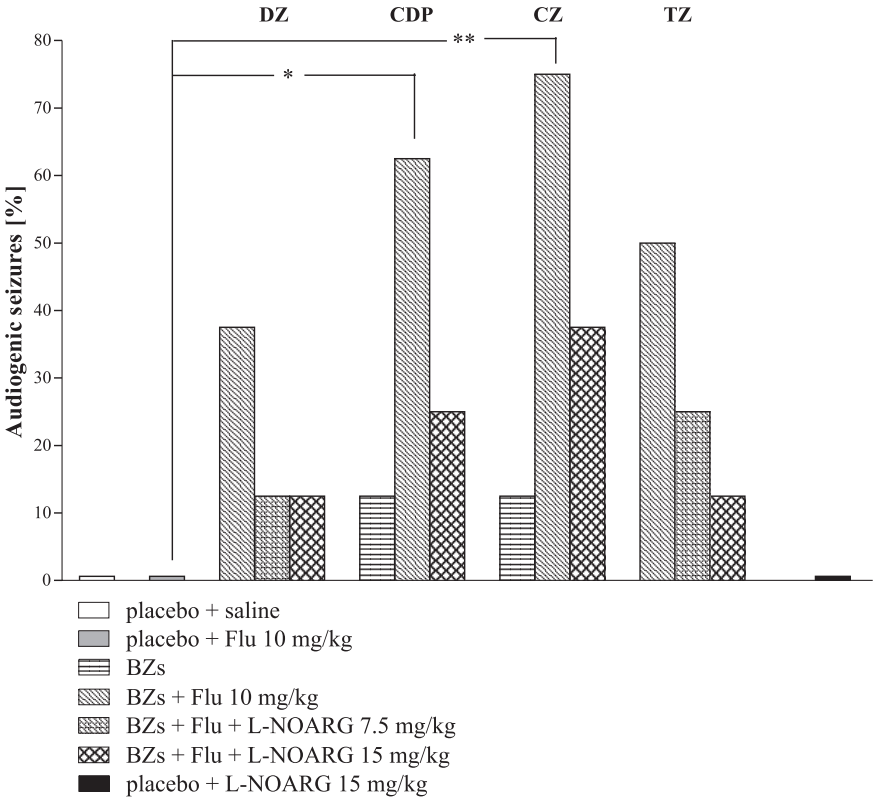

Fig. 3. The influence of L-NOARG (7.5 and 15 mg/kg) on audiogenic seizures during flumazenil (Flu)-induced withdrawal from the following benzodiazepines (BZs): diazepam (DZ), chlordiazepoxide (CDP), clonazepam (CZ) and temazepam (TZ). The results are expressed as the means ± SEM for n = 10 rats. The mean value for the audiogenic seizures in rats implanted with placebo pellets was assumed to be 100%. * $p < 0.05$, ** $p < 0.01$ (χ^2 test with Yates correction)

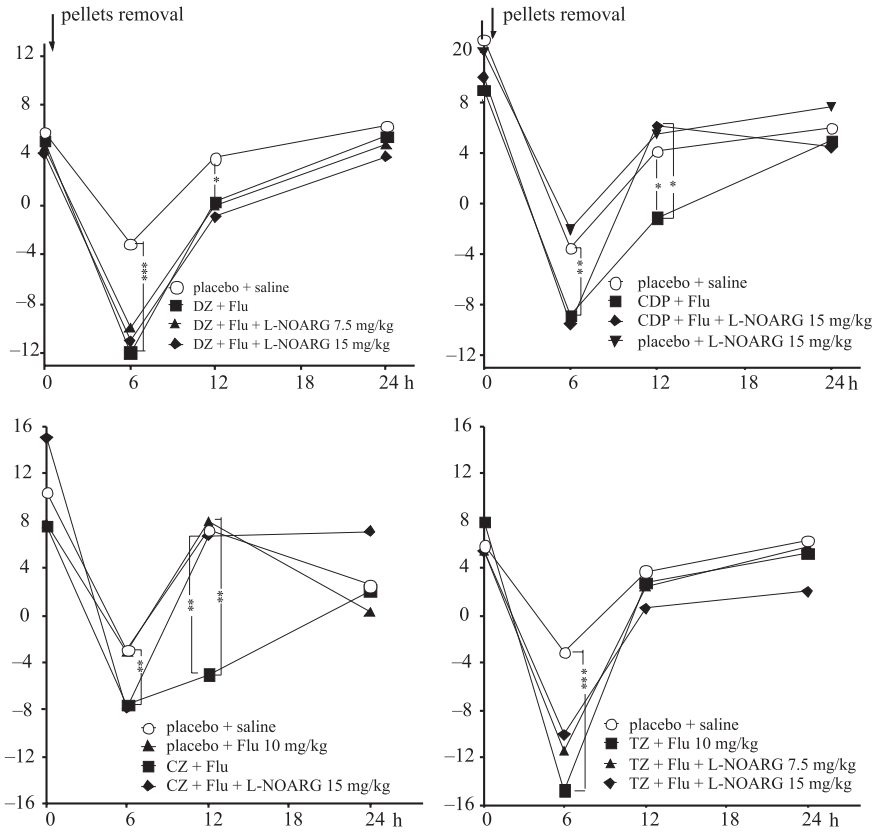

Fig. 4. The influence of L-NOARG (7.5 and 15 mg/kg) on weight loss during flumazenil (Flu)-induced withdrawal from diazepam (DZ), chlordiazepoxide (CDP), clonazepam (CZ) and temazepam (TZ). The results are expressed as the means ± SEM of the body weight difference (in g) between benzodiazepine- and placebo-treated rats (n = 10). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (*post-hoc* Tukey-Kramer's test)

animals, withdrawal-induced changes include spontaneous seizures, increased anxiety, weight loss, hypermotility, tremors and increased sensitivity to audiogenic seizures [5, 27].

In the current study, the development of diazepam dependence in mice was produced by a 14-day exposure to *sc* implanted diazepam-containing pellets in combination with *sc* injected diazepam. In rats, dependence was induced by *sc* implantation of diazepam-, chlordiazepoxide-, clonazepam- and temazepam-containing pellets for 21 days. The *sc* implantation of pellets containing various benzodiazepines has been effectively employed as an animal model for the study of tolerance and physical dependence [27]. The slow release of benzodiazepines from the pellets results in the maintenance of mostly stable plasma levels of the parent benzodiazepines and any metabolites. Thus, it is thought that this method of chronic administration circumvents the rapid metabolism of benzodiazepines and results in continuous occupation of the benzodiazepine recognition site. The appearance of the withdrawal syndrome is widely accepted as confirming the development of physical dependence, and the severity of the withdrawal syndrome indicates the magnitude of the physical dependence [8]. Therefore, the development of physical dependence can be ascertained by precipitating and measuring withdrawal reactions.

In the present experiments, the measured sign of diazepam withdrawal after the removal of the pellets from mice was the intensification of the seizures induced by concomitant application of flumazenil (a benzodiazepine receptor antagonist) and the subthreshold dose of pentetrazole. It is known that administration of flumazenil in animals previously exposed to repeated doses of benzodiazepines abruptly blocks the agonist activity at the receptor and, depending upon the experimental conditions, can elicit an intense withdrawal reaction [24, 25, 60]. The signs of withdrawal in rats were precipitated by administration of flumazenil and manifested as a significant increase in locomotor activity (in rats treated with all of the benzodiazepines used in the experiments), the appearance of audiogenic seizures (in diazepam- and chlordiazepoxide-treated rats) and a reduction in body weight gain at 6 h (for all benzodiazepine-treated groups) and 12 h (in case of diazepam, chlordiazepoxide and temazepam) after removal of the pellets. The apparent discrepancies in the expression of these different withdrawal signs in rats treated with various benzodiazepines used in our study do not seem to be associated with the half-lives of the various benzodiazepines but rather seem to be due to overall experimental variability. The present results confirm findings from the literature [60] showing that the half-lives of benzodiazepines do not play an important role in the severity of the signs of withdrawal.

Although the central benzodiazepine receptor recognition site on the $GABA_A$ receptor plays a key role in the therapeutic effects of benzodiazepines [3, 60], the mechanisms involved in benzodiazepine withdrawal are not fully understood. Some authors have reported alterations in $GABA_A$ receptor subunit expression [35, 36, 50, 58, 61], whereas the others showed changes in $GABA_A$ receptor density [for ref. see 2]. A number of studies demonstrate that adaptive changes in the GABAergic system are difficult to investigate and may be only one of the mechanisms underlying the signs of benzodiazepine withdrawal [20]. Stephens [44] suggests that chronic treatment with benzodiazepines may induce excitatory signaling in systems such as the glutamatergic system as part of a compensatory effect. Thus, it is possible that an overactive glutamatergic system, which may be associated with alterations in NMDA receptor subunit expression or changes in binding to this receptor, may be responsible for the appearance of the benzodiazepine withdrawal signs [17, 44, 51]. The mechanisms underlying these phenomena are now recognized to be complex and to involve multiple other chemical messengers that contribute to the processes associated with substance dependence. One of the more important additional messengers is NO, a key second messenger in the central and peripheral nervous systems that participates in a number of physiological and pathophysiological processes [15, 31]. The results of some studies indicate that the NO pathway may be involved in behavioral changes and neuronal adaptation following the administration of various abused substances. Inhibition of NO production has been shown to attenuate or abolish morphine withdrawal syndrome [10, 32, 57]. It was also observed that administration of L-NAME and 7-nitroindazole, which are NO synthase inhibitors, suppresses several behavioral signs of ethanol withdrawal such as hyperactivity, tremors and audiogenic seizures [1, 54]. Some authors have also demonstrated an inhibitory action of NO synthase inhibitors on precipitated nicotine withdrawal syndrome manifested by weight loss, decreases in motor activity, diarrhea and irritability in rats [19, 39].

Although there is evidence for interactions between the NO and benzodiazepines/$GABA_A$ systems in the central nervous system [13, 30, 56], studies that have evaluated the role of NO in benzodiazepine dependence are limited. Thus, the current studies were undertaken to investigate the effect of the NO synthase inhibitors L-NAME and L-NOARG on the expression of benzodiazepines withdrawal signs in mice and rats.

The major finding of our experiments is that the administration of L-NAME and L-NOARG, which are nonselective NO synthase inhibitors, significantly attenuates pentetrazole-induced withdrawal syndrome in diazepam-dependent mice. The clearest effects were obtained after the application of L-NOARG. This drug evoked a significant decrease in the number of all measured incidents (clonic seizures, tonic convulsions and deaths). Interestingly, total protection against lethality was observed after administration of both NO synthase inhibitors in diazepam-dependent mice, but, in the case of L-NAME, the changes were not significant. Administration of L-NOARG in benzodiazepine-dependent rats also significantly suppressed hypermotility in diazepam- and clonazepam-dependent rats and inhibited the decrease in body weight at 12 h in chlordiazepoxide- and clonazepam-dependent rats. Moreover, L-NOARG protected the benzodiazepines-dependent rats against audiogenic seizures; however, this change was not significant. The reason for the diversity we observed in the effects of the NO synthase inhibitors on withdrawal from various benzodiazepines is unclear and requires further study. It is possible that frequency of audiogenic seizures was an insufficiently precise measure of withdrawal to distinguish the effects of NO synthase. Other investigators have observed similarly diverse results regarding the role of NO in withdrawal from benzodiazepines. For example, it was shown that the inhibition of NO synthase by L-NOARG did not prevent diazepam [33] and clonazepam [34] withdrawal-induced hyperexcitability in the electroshock model but did prevent diazepam [33] and clonazepam [34] withdrawal-induced hypersusceptibility to pentetrazole. In addition, administration of L-arginine, an NO donor, raised the seizure threshold in the electroshock model significantly when co-administered with diazepam [33] and clonazepam [34] but had no effect on diazepam [33] and clonazepam [34] withdrawal-induced hypersusceptibility to seizure in the pentetrazole model [34, 52].

It is difficult to explain precisely which mechanisms are involved in the observed effect of NO synthase inhibitors on the signs of benzodiazepine withdrawal. It is possible that the interactions between the glutamatergic system and NO play some role in these phenomena.

NO plays several physiological roles in the brain, including modulation of either the release or uptake of several neurotransmitters, such as glutamate [14, 41]. Moreover, it is generally known that NO is an intermediate in the signaling provoked by glutamate. Activation of NMDA receptors increases intracellular Ca^{2+} in the postsynaptic neuron, leading to calcium binding to calmodulin and the activation of NO synthase, which stimulates the formation of NO in several brain regions [11, 16]. Furthermore, it has been found that there is reciprocal regulation between glutamate and NO; thus, NO and glutamate may enhance their mutual release and production [22]. Recent studies have shown a clear role for glutamate neurotransmission in the development of dependence produced by the administration of a variety of psychoactive drugs. For example, it was shown that blockade of NMDA receptors markedly reduced opioid withdrawal syndrome [10, 16]. There are also reports demonstrating the inhibitory effects of various NMDA receptor antagonists on ethanol [12, 21] and nicotine [18] withdrawal signs in rodents. Furthermore, other studies have suggested important roles for NMDA receptors and excitatory amino acid stimulation in the signs of benzodiazepine withdrawal. For example, some authors have demonstrated potent suppression of several diazepam withdrawal signs by NMDA receptor antagonists [43, 45, 53] and an upregulation in the protein expression of NMDA receptor subunits in diazepam-withdrawn rat cerebral cortex [51]. Therefore, it is likely that the decrease in GABA inhibition that occurs during withdrawal could lead to the enhancement of the excitatory tone and thereby to the activation of NO synthase, with subsequent release of NO. This mechanism could explain, at least in part, the attenuation of the expression of some of the signs of diazepam withdrawal by NO synthase inhibitors observed in our experiments.

In conclusion, the results of the present study show that a decrease in the level of NO provoked by NO synthase inhibitor administration exerts an inhibitory effect on the some of the signs of benzodiazepine withdrawal in mice and rats. These data implicate NO signaling in the physical dependence produced by benzodiazepines. However, further studies are needed to clarify the precise mechanism underlying our findings.

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