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Role of nitric oxide in the development of tolerance to diazepam-induced motor impairment in mice

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Abstract:
Chronic treatment with the benzodiazepines is well known to produce tolerance, which has been extensively documented to be attributed to modifications in the γ-aminobutyric acid (GABA)ergic neurotransmission. However, literature data have also suggested the participation of different neurotransmitter systems, including glutamatergic, in benzodiazepine tolerance. The purpose of the present study was to determine the role of nitric oxide (NO) in the development of tolerance to the motor dysfunction induced by chronic administration of diazepam. The motor performance was assessed on the 1st and 10th day of experiment, using the rotarod and chimney tests in mice. Treatment of animals with both non-selective NO synthase (NOS) inhibitors: N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NOARG) and selective NOS inhibitor: 7-nitroindazole was able to prevent the development of tolerance to the motor impairing effect of diazepam. Moreover, administration of L-arginine, a NO precursor, facilitated the development of diazepam-induced tolerance in rotarod test. These findings suggest that NO may be involved, at least in part, in the tolerance to the motor dysfunction, developed during the chronic administration of diazepam in mice.

Key words:
nitric oxide, diazepam, tolerance, motor impairment, mice

Introduction

The benzodiazepines are a group of psychoactive drugs that exert a number of pharmacological effects, such as anxiolysis, sedation, hypnosis, anterograde amnesia, muscle relaxation and anticonvulsant activity. They do so by binding to the central benzodiazepine receptor recognition site on the γ-aminobutyric acid $(GABA)_A$ receptor complex and potentiating the inhibitory effect of GABA [45, 56]. There is a line of evidence indicating that a long-term administration of benzodiazepines results in the development of tolerance to some effects of these drugs (including their sedative, muscle relaxant and anticonvulsant effects), and this phenomenon limits their clinical efficacy. Such treatment, even at therapeutic doses, is also associated with the development of physical dependence [10, 36]. The molecular bases for tolerance to benzodiazepines still remain unclear. However, tolerance and dependence to benzodiazepines appear not related to the pharmacokinetic mechanisms of these drugs [5, 14, 17, 49]. It has been established that tolerance to benzodiazepines is associated with an adaptive process leading to progressive diminution in the activity of the drug at the $GABA_A$ receptor complex

[2, 13]. It has also been hypothesized that sensitization of excitatory mechanisms (including the glutamatergic system) may be a part of compensatory mechanisms to benzodiazepine-induced chronic enhancement of GABAergic inhibition [2, 41].

There is evidence that nitric oxide (NO), an intracellular and short-lasting retrograde messenger, is involved in different peripheral and central functions [7, 9, 19, 48]. Among a number of physiological processes in the central nervous system (CNS), such as control of sleep [30], synaptic plasticity [32], learning and memory formation [57], it has been shown that NO can also participate in the mechanisms of drug tolerance and dependence [4, 22, 28, 52, 55].

NO is synthesized from L-arginine in several tissues by a reaction catalyzed by NO synthase (NOS), which is found in three distinct isoforms: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS). Endothelial cells and neuronal tissues contain constitutively expressed NOS isoforms, which are Ca^{2+}/calmodulin-dependent, whereas inducible NOS is an isoform produced in macrophages and other cell types and is Ca^{2+}-independent. Although all forms can be found in the CNS, because of the temporal and spatial properties of this tissue, the specific actions on neurotransmission may be attributed primarily to NO produced by nNOS located in neurons [29]. It has been observed that nNOS produced NO almost exclusively following activation of N-methyl-D-aspartate (NMDA) receptors [15] and has the most crucial role in mediating drug tolerance and dependence among all NOS isoforms [54].

An increasing body of evidence suggests an interaction between NO and GABA, a neurotransmitter which is closely connected with the effects of benzodiazepines. It has been reported that NO was able to modulate release of glutamate and GABA in the dorsal striatum [47] and in the nucleus accumbens [24]. Moreover, histochemical mapping of NOS revealed that NOS-positive neurons were co-localized with GABA or GABA receptor in several brain regions [53, 58]. *In vivo* and *in vitro* studies suggest that NO modulates either release or uptake of GABA and the activity of $GABA_A$ receptor or acts directly on $GABA_A$ receptor [12, 18, 26, 38, 59]. Furthermore, several studies have implicated NO-dependent pathways of the CNS in the effects of benzodiazepines, using acute protocols. It has been shown that inhibition of NOS prolonged the sleeping time induced by benzodiazepines [42], enhanced the anticonvulsant [43], antinociceptive [44] and anxiolytic [33] effect of benzodiazepines. However, there are limited data obtained with the use of chronic protocols, concerning NO and benzodiazepine relationship.

The current study was undertaken to determine the involvement of the NO in the development of tolerance to diazepam-induced motor impairment. This was done by measuring motor coordination in diazepam-administered mice after chronic treatment with L-NAME and L-NOARG, nonselective inhibitors of the NOS isoforms, 7-nitroindazole, a preferential inhibitor of nNOS [3] and L-arginine, a substrate for NO formation.

Materials and Methods

Animals

The experiments were carried out on male albino Swiss mice weighing 20–25 g at the beginning of the experiment. The animals were housed in groups of ten and maintained on a 12 h light-dark cycle at controlled temperature (21°C). They received standard rat diet and tap water *ad libitum*. All behavioral experiments were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and to the European Community Council Directive for the Care and Use of Laboratory Animals of 24 November 1986 (86/609/EEC), and approved by the local ethics committee.

Drugs and tolerance procedure

N^G-nitro-L-arginine methyl ester (L-NAME, Sigma, USA), N^G-nitro-L-arginine (L-NOARG, Sigma, USA), L-arginine (Sigma, USA) were dissolved in 0.9% saline. 7-Nitroindazole (RBI, USA) was suspended in a few drops of Tween-80 and then dissolved in 0.9% saline. Diazepam (Relanium, Polfa, Poland) was diluted in 0.9% saline. Control animals were injected with the corresponding vehicle.

Tolerance to diazepam-induced motor impairment was induced by repeated (10 days), subcutaneous (*sc*) administration of diazepam (5 mg/kg/day). This dose of diazepam was chosen from the literature data, showing the development of tolerance during the chronic administration of diazepam [23, 31, 40]. L-NAME

(50, 100 mg/kg), L-NOARG (10, 20 mg/kg), 7-nitroindazole (10, 20 mg/kg) and L-arginine (125, 250 mg/kg) were injected intraperitoneally (*ip*). The doses of L-NAME, L-NOARG, 7-nitroindazole and L-arginine, were tested in our previous experiments (data not published) and those which did not affect the motor performance in mice were used in these experiments. All substances were administered in an injection volume of 10 ml/kg.

Behavioral tests

The motor coordination of mice was measured on the 1st and 10th day of the experiment, using the rotarod test and the chimney test.

Rotarod test

The test was performed according to the method of Dunhann and Miya [8]. The mice were trained and tested using a bar rotating at a constant speed of 18 rpm (2 cm in diameter). Before drug testing, the mice were trained daily for a 3-day period. For each training session the mice were placed on a rotating rod for 3 min with a unlimited number of trials. Drug testing was conducted at least 24 h after the final training trial. During the test the mice had to remain on the rod for as long as they could. The length of time that the animal remained on the rod was recorded (a 60 s maximal trial was used for the test).

Chimney test

The animals had to climb backwards up a plastic tube (3 cm in inner diameter, 25 cm long). The mice were trained once daily for 3 days. Motor impairment was assessed as the inability of mice to climb backwards up the tube within 60 s. The length of time that the mice spent in the chimney was recorded [6].

Pretreatment times were 30 min for diazepam and 35 min for L-NAME, L-NOARG, 7-nitroindazole and L-arginine.

Statistical analysis

Results in these experiments were analyzed by one-way ANOVA. *Post-hoc* comparisons were carried out by Tukey-Kramer test. A level of $p < 0.05$ was considered as statistically significant. Data are presented as the mean ± SEM.

Results

Effects of diazepam on performance in the rotarod test (Fig. 1A–4A) and chimney test (Fig. 1B–4B)

The repeated (10 days) treatment of mice with diazepam (5 mg/kg/day) resulted in the development of tolerance to its motor impairing effect, which was observed both in the rotarod test and the chimney test and manifested by statistically significant differences between the acute diazepam-treated group (1st day of the experiments) and chronically diazepam-treated mice (10th day of the experiments).

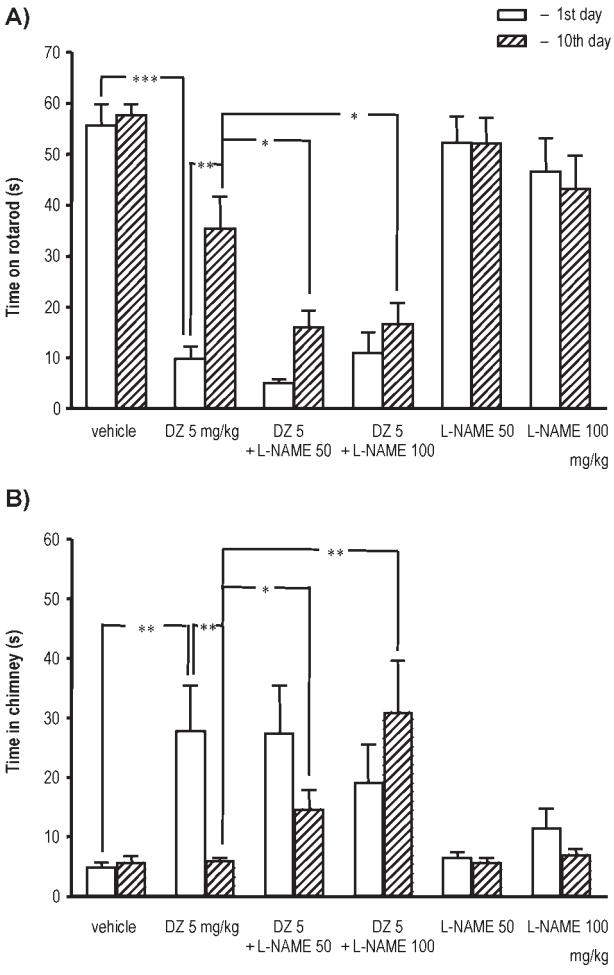

Fig. 1. Effects of L-NAME (50, 100 mg/kg *ip*) on the development of tolerance to the motor impairing effect of diazepam (DZ, 5 mg/kg *sc*), measured by the rotarod test **(A)** and chimney test **(B)**. Diazepam was injected 30 min before the test, L-NAME or saline were injected 5 min before the diazepam administration. Results are expressed as the mean ± SEM (n = 8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to appropriate control (Tukey-Kramer's test)

The influence of L-NAME on the development of tolerance to diazepam-induced motor impairment in the rotarod test (Fig. 1A) and chimney test (Fig. 1B)

Administration of diazepam (5 mg/kg) at a single dose on the 1st day of the experiment impaired the motor coordination of mice. This effect was observed both in the rotarod test ($p < 0.001$) and in the chimney test ($p < 0.01$). There were no significant effects of acute L-NAME (50, 100 mg/kg) pretreatment on the diazepam-induced motor impairing effect, as measured by the rotarod and the chimney tests on the 1st day of the experiment. However, L-NAME at a dose of 50 or 100 mg/kg/day, coadministered with diazepam, prevented the development of tolerance to the motor impairment effect of diazepam, both in the rotarod test ($p < 0.01$) and dose-dependently in the chimney test ($p < 0.05$, $p < 0.01$, respectively) as measured on the 10th day of the experiment. L-NAME (50 or 100 mg/kg), given alone at a single or repeated (for 10 days) doses, had no significant effect on the motor performance measured by the rotarod and chimney tests.

The influence of L-NOARG on the development of tolerance to diazepam-induced motor impairment in the rotarod test (Fig. 2A) and the chimney test (Fig. 2B)

Co-administration of L-NOARG with diazepam (5 mg/kg) at a single dose (1st day of the experiment) did not affect the diazepam-induced motor impairing

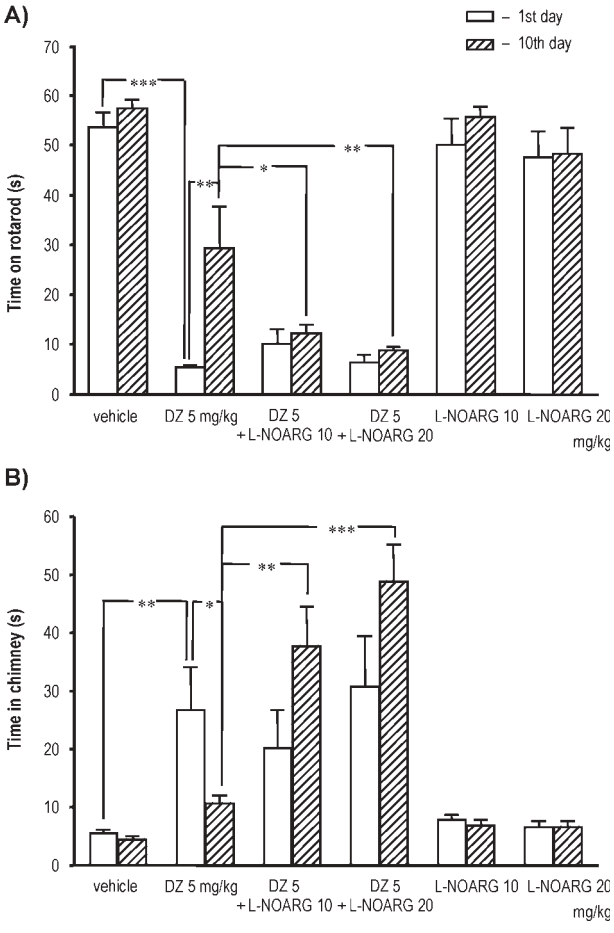

Fig. 2. Effects of L-NOARG (10, 20 mg/kg *ip*) on the development of tolerance to the motor impairing effect of diazepam (DZ, 5 mg/kg *sc*), measured by the rotarod test **(A)** and chimney test **(B)**. Diazepam was injected 30 min before the test, L-NOARG or saline were injected 5 min before the diazepam administration. Results are expressed as the mean ± SEM (n = 8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to appropriate control (Tukey-Kramer's test)

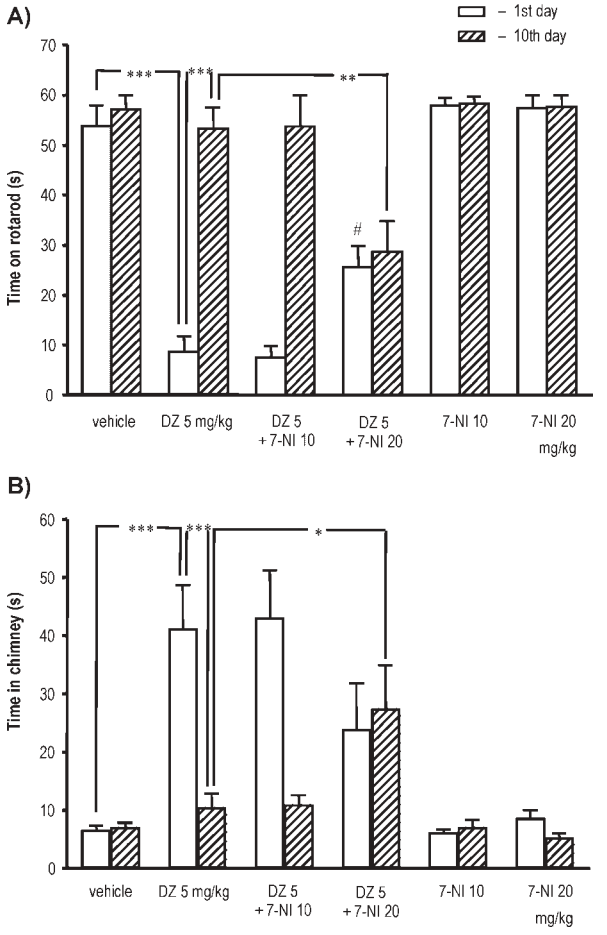

Fig. 3. Effects of 7-nitroindazole (7-NI, 10, 20 mg/kg *ip*) on the development of tolerance to the motor impairing effect of diazepam (DZ, 5 mg/kg *sc*), measured by the rotarod test **(A)** and chimney test **(B)**. Diazepam was injected 30 min before the test, 7-nitroindazole or saline were injected 5 min before the diazepam administration. Results are expressed as the mean ± SEM (n = 8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to appropriate control, # $p < 0.01$ compared to diazepam on the 1st day (Tukey-Kramer's test)

effect, as measured by the rotarod test and chimney test. The repeated pretreatment of L-NOARG at the doses of 10 and 20 mg/kg with diazepam dose-dependently inhibited the development of diazepam-induced tolerance both in the rotarod test ($p < 0.05$, $p < 0.01$, respectively) and in the chimney test ($p < 0.01$, $p < 0.001$, respectively). There was no significant effect on the motor coordination of mice, measured by the rotarod test and the chimney test, following acute and chronic L-NOARG (10 and 20 mg/kg) injection alone.

The influence of 7-nitroindazole on the development of tolerance to diazepam-induced motor impairment in the rotarod test (Fig. 3A) and the chimney test (Fig. 3B)

Administration of 7-nitroindazole with diazepam (5 mg/kg) at a single dose of 20 mg/kg (1st day of the experiment) decreased the diazepam-induced motor impairment ($p < 0.05$), in the rotarod test, but not in the chimney test. The lower dose of 7-nitroindazole (10 mg/kg) had no significant effect on the motor dysfunction caused by diazepam. The chronic pretreatment of 7-nitroindazole at a dose of 20 mg/kg with diazepam (5 mg/kg) resulted in the inhibition of the development of diazepam-induced tolerance to its motor impairing effect. This effect was observed both in the rotarod test ($p < 0.01$) and in the chimney test ($p < 0.05$). Chronic administration of the lower dose of 7-nitroindazole (10 mg/kg) had no significant effects on the diazepam-induced tolerance to the motor incoordination. The acute or repeated (for 10 days) administration of 7-nitroindazole alone at the doses of 10 and 20 mg/kg had no significant effect on the motor performance measured by the rotarod and chimney tests.

The influence of L-arginine on the development of tolerance to diazepam-induced motor impairment in the rotarod test (Fig. 4A) and the chimney test (Fig. 4B)

Co-administration of L-arginine with diazepam (5 mg/kg) at an acute dose did not affect the diazepam-induced motor impairing effect, as measured by both the rotarod test and the chimney test on the 1st day of the experiment. The chronic pretreatment of L-arginine at the doses of 125 and 250 mg/kg facilitated the development of diazepam-induced tolerance to the motor incoordination of mice. This effect was observed in the rotarod test ($p < 0.01$ for both doses), but not in the chimney test. There were no significant effects on the motor coordination of mice, measured by the rotarod test and the chimney test, following acute and chronic L-arginine (125 and 250 mg/kg) injection alone.

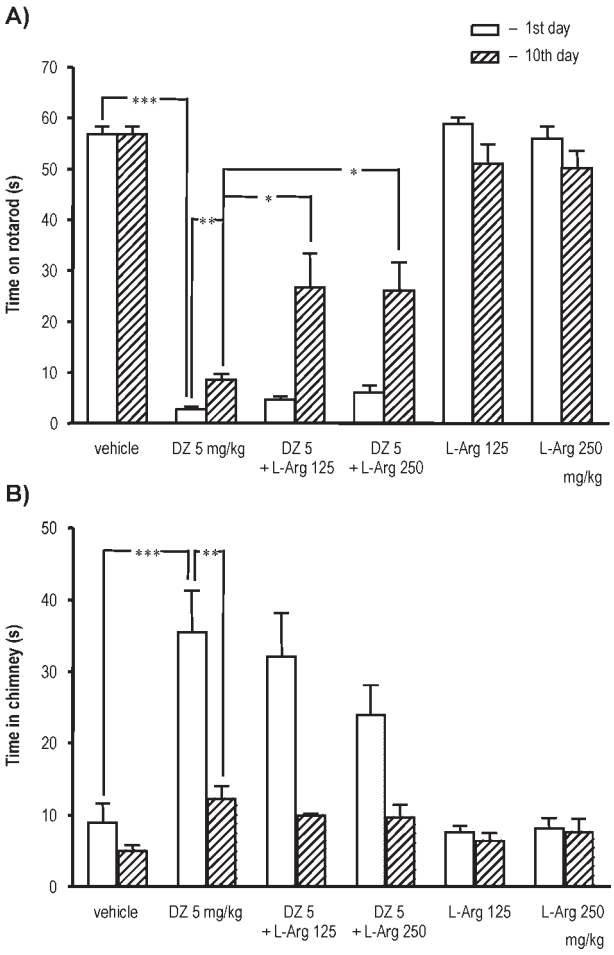

Fig. 4. Effects of L-arginine (L-Arg, 125, 250 mg/kg *ip*) on the development of tolerance to the motor impairing effect of diazepam (DZ, 5 mg/kg *sc*), measured by the rotarod test **(A)** and chimney test **(B)**. Diazepam was injected 30 min before the test, L-arginine or saline were injected 5 min before the diazepam administration. Results are expressed as the mean ± SEM (n = 8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to appropriate control (Tukey-Kramer's test)

Discussion

Tolerance to benzodiazepines has been reported in various species although the degrees of tolerance and time course have varied markedly. Tolerance develops after both low doses and high doses if the frequency and duration of administration are sufficient [10, 16]. It is known that this slowly developing tolerance after

chronic treatment with benzodiazepine receptor agonists, such as diazepam, lorazepam and flurazepam is a results of downregulation of $GABA_A$ receptor complex [25, 35], changes in $GABA_A$ receptor subunit gene expression [25, 37], functional allosteric uncoupling of the benzodiazepine receptor recognition site for $GABA_A$ receptors [1, 46], and decreased coupling between the benzodiazepine site and GABA receptor-gated chloride channels [1]. However, a reduction in the effect of benzodiazepines at the $GABA_A$ receptor complex does not seem to be the only mechanism involved in the development of benzodiazepine tolerance. For example, a compensatory increase in the excitatory glutamatergic response, named an oppositional response, has also been put forward as a means for explaining this phenomenon [2, 41].

The present studies showed that repeated (5 mg/kg/day *sc* for 10 days) administration of diazepam led to the development of tolerance to its motor impairing effect, both in the rotarod and chimney test. The major findings of the current study showed that L-NOARG and L-NAME, nonselective NOS inhibitors, prevented the development of tolerance to the motor impairing effect of diazepam. It is known that L-NAME and L-NOARG are nonselective NOS inhibitors that, besides its central activity, affect also the cardiovascular system and increase arterial blood pressure [34] which then may affect the excitability of the central neurons. In order to avoid the effect of arginine-derived NOS inhibitors on blood pressure and muscarinic receptor, we used 7-nitroindazole, an inhibitor of neuronal NOS [3]. We have observed that 7-nitroindazole, was also able to inhibit the development of tolerance to diazepam, at higher dose of 20 mg/kg. The lower dose of 7-nitroindazole (10 mg/kg) failed to affect the development of diazepam-induced tolerance. It is pertinent to note that in the present study the acute or chronic administration of a NOS inhibitor alone did not affect the motor performance of mice, because we used a dose range below that needed to cause motor deficit. Additionally, in the present study we have observed an inhibiting effect of a higher dose of 7-nitroindazole on diazepam-induced motor deficit, measured by rotarod test on the 1st day of the experiment. It is difficult to explain and further investigation must be undertaken to clarify this interesting effect.

Another interesting observation arising from the present study was the facilitation of development of diazepam-induced tolerance to motor impairing effect in the rotarod test after chronic pretreatment with L-arginine, an endogenous NO precursor, in combination with diazepam. Acute or chronic L-arginine alone had no motor impairing effect, both in the rotarod and in the chimney test. The lack of effect of L-arginine on the tolerance to diazepam in the chimney test is difficult to explain. Some studies have shown that L-arginine up to 1000 mg/kg was effective without impairing open-field locomotor activity in mice [50, 51]. Therefore, it is possible that too low, inefficient doses of the NO precursor which was used in our experiments (up to 250 mg/kg) would account for the lack of effect of L-arginine on the tolerance to diazepam in the chimney test. However, clear effect of L-arginine in the rotarod test seems to confirm the role of NO in the development of tolerance to motor impairing effect of diazepam.

The involvement of the NO system in tolerance phenomenon and the effects of NO synthase inhibitors on adaptive mechanisms related to dependence on drugs have been the subject of numerous studies in which controversial results were obtained. For example, it was shown that the inhibition of NOS by L-NAME, L-NOARG and 7-nitroindazole blocked the rapid development of tolerance to the motor impairment and hypothermia induced by ethanol [21, 22, 55]. Moreover, the blockade of NOS also affected adaptive mechanisms associated with dependence on other drugs, such as the sensitization to nicotine [39], cocaine or methamphetamine [20] or the development of tolerance to morphine [27, 28]. But still there is no so many investigations which determine the role of NO in the development of tolerance to benzodiazepines. Nidhi et al. [31] showed that L-NOARG did not prevent the development of tolerance to the anticonvulsant activity of diazepam in rats. Furthermore, they observed that L-arginine, a donor of NO, was able to inhibit tolerance to diazepam anticonvulsant effect.

These discrepant results imply that processes leading to the development of tolerance to different behavioral effects of benzodiazepines may involve distinct mechanisms which may be differentially manipulated. For example, it has been suggested that the mechanisms underlying tolerance to the anxiolytic effects of diazepam may be different from that underlying tolerance to sedation [11].

The mechanisms by which NOS inhibitors affect benzodiazepine tolerance are complex and not fully understood. It is presumed that chronic treatment with NOS inhibitors and diazepam would lead to prevention of $GABA_A$ receptor down-regulation and/or glu-

tamate receptor up-regulation. This possible mechanism could explain the inhibition of diazepam-induced tolerance by NOS inhibitors, observed in our experiments. On the contrary, administration of L-arginine, a donor of NO, with diazepam could facilitate up-regulation of NMDA receptors and consequently simplify the development of diazepam tolerance. However, further studies are required to clarify the precise mechanisms underlying our findings, because the presence of other interactions in the CNS could not be excluded.

In conclusion, our results show that both nonselective NOS inhibitors (L-NAME, L-NOARG) and selective nNOS inhibitor (7-nitroindazole) can prevent the development of tolerance to the motor impairing effect of diazepam. The present study also demonstrates that L-arginine, a donor of NO, is able to facilitate the development of tolerance to diazepam in the rotarod test. Furthermore, our findings suggest that NO may play some role in the mechanisms of diazepam-induced tolerance to its motor impairing effect in mice.

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