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Effects of NOS inhibitors and a NO precursor on the expression of tolerance to diazepam-induced motor impairment in mice

Wpływ inhibitorów NOS i prekursora NO na ekspresję tolerancji na działanie zaburzające koordynację ruchową diazepamu u myszy

Benzodiazepines (BZ) are anxiolytic, anticonvulsant, sedative and hypnotic compounds usually prescribed on a long-term therapy. However, chronic treatment with these compounds induces tolerance [4,8,27]. All BZ have the capacity to promote the binding of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) to the $GABA_A$ receptors. $GABA_A$ receptors exist as multisubunit (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, φ, π and ρ_{1-3}), ligand-gated chloride channels [8,27]. Tolerance to BZ's diverse effects (sedative, muscle relaxant, anxiolytic and anticonvulsant) after a prolonged treatment is a well-documented issue [2, 4]. Nevertheless, the mechanism of development of tolerance to the action of BZ is basically unknown [2,4,8]. There is evidence for $GABA_A$ receptors downregulation following chronic BZ administration [12] and for changes in the various subunits $GABA_A$ receptor [4]. Another concept that has been evolved is that excitatory mechanisms (a likely candidate is the glutamatergic system) become more sensitive as a part of compensatory mechanisms to BZ-induced chronic enhancement of GABAergic inhibition [2].

Nitric oxide (NO), a free gaseous signalling molecule, is involved in the regulation of the cardiovascular, nervous and immune system [6,7]. It is synthesized from L-arginine by a nitric oxide synthase (NOS)-catalysed reaction [7]. There are three genetically different isoforms of NOS which account for NO production. They include neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). Of the three NOS isoforms, nNOS constitutes the predominant source of NO in neurons and localizes to synaptic spines [7]. In addition, it has been observed that nNOS produces NO almost exclusively following activation of N-methyl-D-aspartate (NMDA) receptors [9].

NO is supposed to play an important role in several brain functions and/or dysfunctions, including regulation of neuronal excitability, synaptic plasticity, anxiety, seizure activity and drug tolerance [1,9,27,28,32]. It also known that NO regulates the release and uptake of neurotransmitters such as dopamine, serotonin, glutamate and GABA [9,13].

There are data pointing to the relationship between L-arginine: NO:cGMP pathway and GABA-mediated transmission in the central nervous system (CNS). Some studies described co-localization

of NO with GABA in the cortex and the spinal cord [30]. Moreover, there is evidence that NO is released by GABAergic neurons of animal cortex [13,21]. It has been recently demonstrated that activation of $GABA_A$ receptor by diazepam (DZ) leads to increased numbers of nNOS cells [17]. There are data indicating that inhibition of NOS prolongs the sleeping time induced by BZ [25], enhances the anticonvulsant [24], antinociceptive [23] and anxiolytic [20] effects of BZ. In addition, there are findings suggesting some role of L-arginine:NO:cGMP in the development of DZ-induced tolerance to its motor impairing effect in mice [26].

In the present work we investigated the effect of compounds which modulate the L-arginine: NO:cGMP pathway, on the expression of tolerance DZ-induced motor impairment in mice.

MATERIAL AND METHODS

Animals

The examinations 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 eight and maintained on a 12 h light-dark cycle at controlled temperature (21°C). They received standard food (Murigan pellets, Bacutil, Motycz, Poland) and tap water *ad libitum*. All behavioural 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 Directive for the Care and Use of Laboratory of 24 November 1986 (86/609/EEC), and approved by the Local Ethics Committee (508/2004)

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. Diazepam (DZ, Relanium, Polfa, Poland) was diluted in 0.9% saline. Control animals were injected with the corresponding vehicle.

Tolerance to DZ-induced motor impairment was induced by repeated (10 days), subcutaneous (*sc*) administration of DZ (5 mg/kg). A dose of DZ was chosen from the literature data, showing the development of tolerance during the chronic administration of DZ [15, 19].

On the 10^{th} day of experiment, mice were pretreated with L-NAME (50, 100 mg/kg, *ip*), L-NOARG (10, 20 mg/kg, *ip*) and L-arginine (125, 250 mg/kg, *ip*) 30 min before injection of DZ (5 mg/kg, *sc*). The doses of L-NAME, L-NOARG and L-arginine were tested in our previous experiments (data not published) and those which did not affect the motor performance in control mice were used in these experiments. All substances were administered in an injection volume of 10 ml/kg.

Behavioral tests

Rotarod test. 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 an unlimited number of trials. All animals of training session were used in the following experiments. 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 the animal remained on the rod was recorded (a 60 s maximal trial was used for the test) [31].

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 [31]. Pretreatment times were 30 min for DZ and 35 min for L-NAME, L-NOARG and L-arginine.

The rotarod and chimney tests are used to evaluate the activity of drugs interfering with motor coordination. However the chimney test (not complicated method) can be used as an additional test with other test determining muscle relaxant activity [31].

S t a t i s t i c a l a n a l y s i s. Results in these experiments were analyzed by one-way ANOVA. Post hoc comparisons were carried out by Tukey-Kramer test. The level of $p<0.05$ was considered as statistically significant. Data are presented as mean ± SEM.

RESULTS

EFFECTS OF DZ ON PERFORMANCE IN THE ROTAROD TEST (FIG. 1A, 2A, 3A) AND CHIMNEY TEST (FIG. 1B, 2B, 3B)

Administration of DZ (5 mg/kg) at a single dose on day 1 impaired the motor coordination of mice. This effect was observed both in the rotarod test and chimney test. The repeated (10 days) treatment of mice with DZ (5 mg/kg) resulted in the development of tolerance to its motor impairing effect, which was observed both in the rotarod and chimney test and manifested by statistically significant differences between the acute DZ-treated group (day 1) and chronically DZ-treated mice (day 10).

THE INFLUENCE OF L-NAME ON THE EXPRESSION OF TOLERANCE TO DZ-INDUCED MOTOR IMPAIRMENT IN THE ROTAROD TEST (FIG.1A) AND CHIMNEY TEST (FIG.1B)

Co-administration of L-NAME in the dose of 100 mg/kg (day 10) with DZ (5 mg/kg, repeated treatment: 10 days) resulted in the inhibition of the expression of DZ-induced tolerance to its motor impairment effect observed, both in the rotarod ($p<0.05$) and chimney ($p<0.01$) test. There were no significant effects on the expression of DZ-induced tolerance to the motor incoordination, following co-administration (day 10) of the lower dose of L-NAME (50mg/kg) with DZ both in the rotarod and chimney test.

THE INFLUENCE OF L-NOARG ON THE EXPRESSION OF TOLERANCE TO DZ-INDUCED MOTOR IMPAIRMENT IN THE ROTAROD TEST (FIG.2A) AND CHIMNEY TEST (FIG. 2B)

Co-administration of L-NOARG in the doses of 10 or 20 mg/kg (day 10) with DZ (5 mg/kg, repeated treatment: 10 days) resulted in the inhibition of the expression of DZ-induced tolerance to its motor impairment effect. This effect was observed both in the rotarod test ($p<0.01$) and the chimney test ($p<0.05$ for 10 mg/kg and $p<0.001$ for 20 mg/kg).

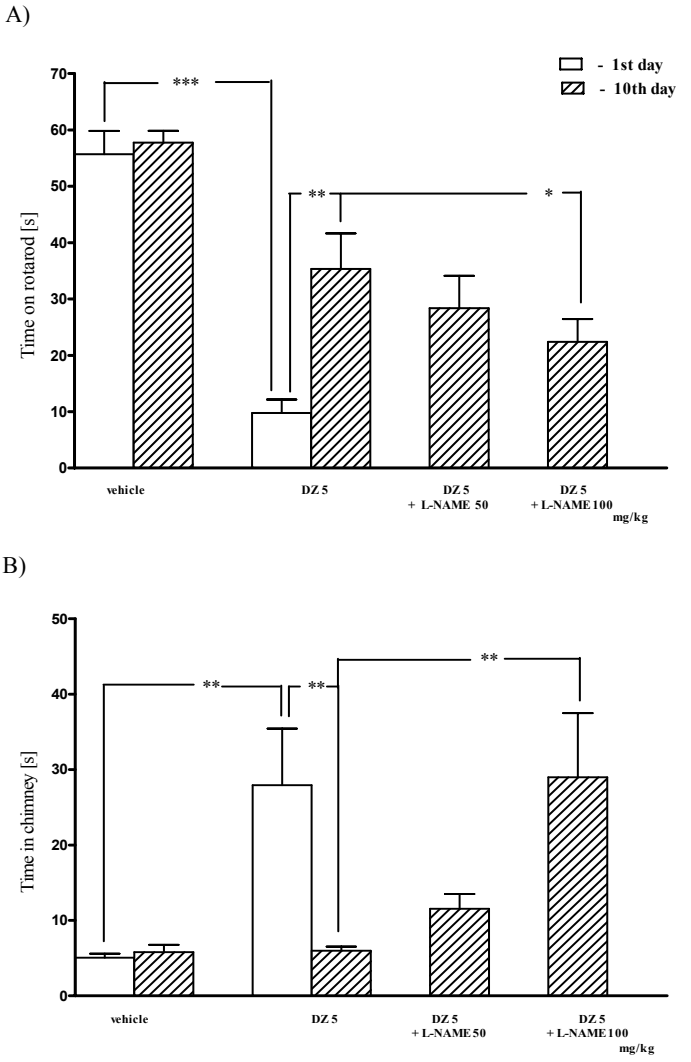


Fig.1. The influence of L-NAME (50 or 100 mg/kg *ip*) on the expression of tolerance to diazepam-induced motor impairment (DZ, 5 mg/kg *sc*), measured by the rotarod test (A) and the chimney test (B). Results are expressed as mean±SEM (n=8 mice/group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to appropriate control (Tukey-Kramer's test)

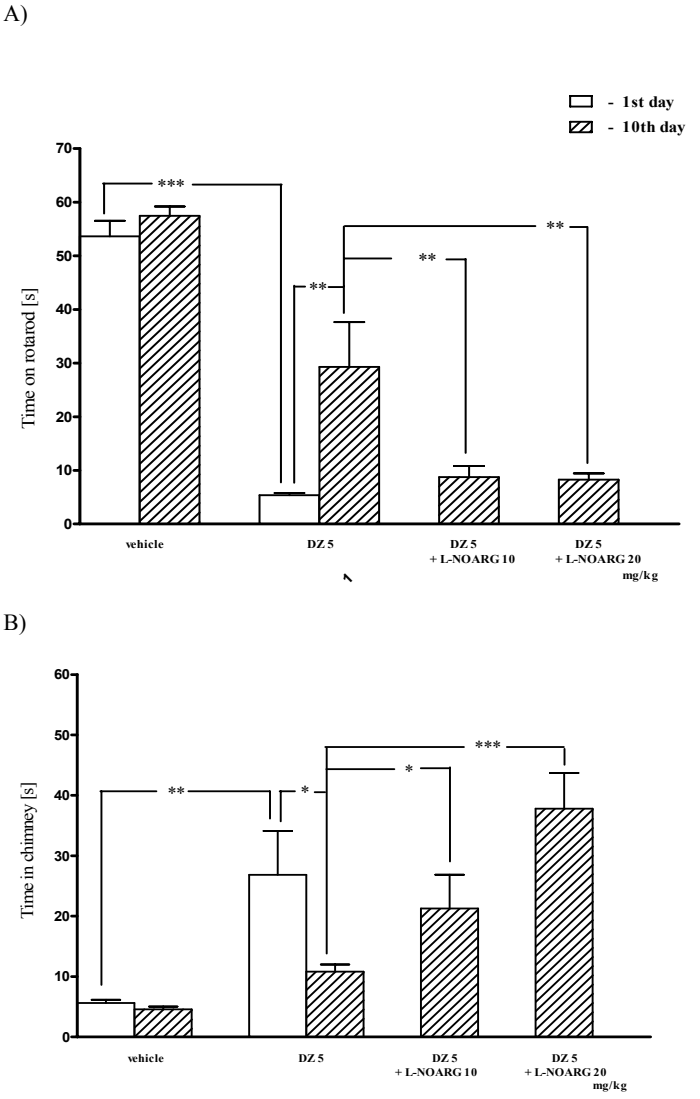
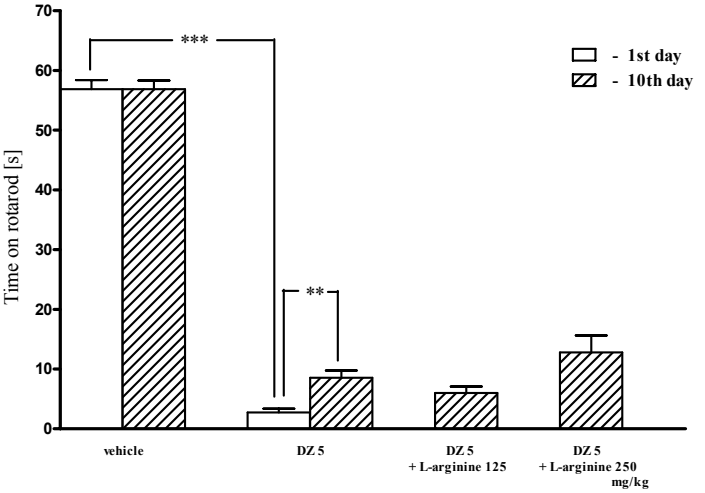


Fig. 2. The influence of L-NOARG (10 or 20 mg/kg ip) on the expression of tolerance to diazepam-induced motor impairment (DZ, 5 mg/kg sc), measured by the rotarod test (A) and the chimney test (B). Results are expressed as mean±SEM (n=8 mice/group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to appropriate control (Tukey-Kramer's test)

THE INFLUENCE OF L-ARGININE ON THE EXPRESSION OF TOLERANCE TO DZ-INDUCED MOTOR IMPAIRMENT IN THE ROTAROD TEST (FIG.3A) AND CHIMNEY TEST (FIG.3B)

Co-administration of L-arginine in the doses of 125 or 250 mg/kg (day 10) with DZ (5 mg/kg, repeated treatment: 10 days) did not affect the expression of tolerance to the DZ-induced motor impairing effect, as measured by the rotarod and chimney test.

A)



B)

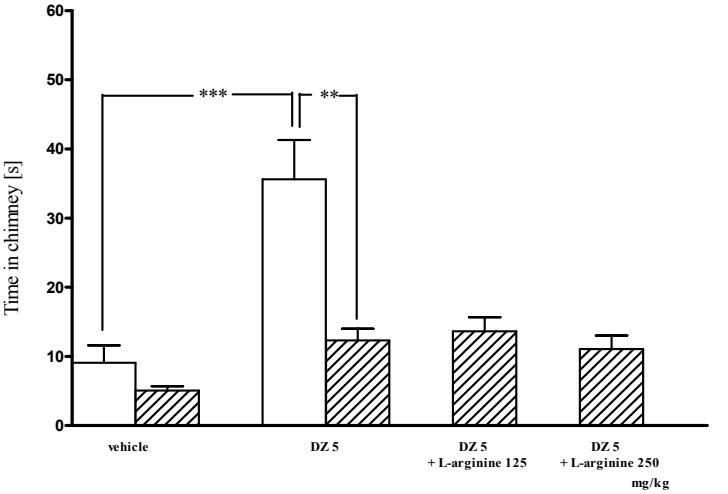


Fig. 3. The influence of L-arginine (125 or 250 mg/kg ip) on the expression of tolerance to diazepam-induced motor impairment (DZ, 5 mg/kg sc), measured by the rotarod test (A) and the chimney test (B). Results are expressed as mean±SEM (n=8 mice/group). ** $p<0.01$, *** $p<0.001$ compared to appropriate control (Tukey-Kramer's test)

DISCUSSION

Our present experiments showed that DZ, a prototypical BZ, administered repeatedly for ten days, in the dose of 5 mg/kg, resulted in the development of tolerance to its motor impairing effect, both in the rotarod and chimney test. Many studies using experimental animals demonstrated the development of tolerance to some pharmacological effects produced by BZ, such as sedative, anxiolytic and anticonvulsant [5, 10, 11, 12, 19]. However, the exact mechanism of this phenomenon is far from being completely understood. It was established that tolerance to BZ was associated with a progressive diminution in the activity of the drug at the $GABA_A$ receptor complex. Numerous studies demonstrated changes in the cortical and hippocampal expression of $GABA_A$ receptor subunits in tolerant animals [2, 4]. In addition, chronic treatment of rats with DZ caused a loss in the ability of DZ to potentiate GABA-stimulated chloride flux [4]. Moreover, it is generally understood that the neurophysiological activity of the mammalian brain is maintained by the balance between inhibitory (such as GABA) and excitatory (such as glutamate) neurotransmission. Regarding this issue, the compensatory increase in the glutamatergic neurotransmission was supposed to be the second model to explain the BZ tolerance [2, 22].

The literature data indicated that NO, an unusual neurotransmitter in the CNS, produced in response to NMDA receptors, is involved in tolerance phenomenon of many psychoactive substances such as opioids [3, 16], ethanol [14, 32], nicotine [29] and psychostimulants [28]. However, there are not so many investigations which determine the role of NO in the tolerance to BZ. Thus, we decided to examine the involvement of NO in the expression of tolerance to DZ-induced motor impairment in mice. In our study, NOS inhibitors (L-NOARG and L-NAME) and an endogenous precursor of NO (L-arginine) were administered prior to the injections of DZ, on the last day of experiment (day 10). The major findings of the present study indicated that L-NOARG (10 and 20 mg/kg) and L-NAME (100 mg/kg) inhibited the expression of tolerance to the motor impairing effect of DZ, both in the rotarod and chimney test. The effect of L-NOARG was dose-dependent. These results correlate with our previous studies showing that L-NAME, L-NOARG and 7-NI were able to prevent the development of tolerance to the motor impairing effect of DZ, both in the rotarod and chimney test [26]. Interestingly, the published data concerning the NO system involvement in tolerance of BZ are controversial. For instance, Nidhi et al. [19] have recently reported that L-NOARG does not prevent the development of tolerance to the anticonvulsant effect of DZ in rats.

Moreover, our present data demonstrated that L-arginine did not change the expression of the tolerance to the DZ-induced motor impairment in mice. These results correlate with our previous studies indicating no effect of L-arginine on the development of tolerance to the motor impairing effect of DZ, both in the rotarod and chimney test [26]. The lack of influence of L-arginine on the tolerance to DZ in these tests is difficult to explain. Some studies showed that L-arginine up to 1000 mg/kg was effective without impairing open-field locomotor activity in mice [28, 29]. Therefore, it is possible that incapable doses (too low) of L-arginine were used in our experiments (up to 250 mg/kg). These doses would account for the lack of effect of the precursor of NO on the expression to DZ-induced motor impairment, both in the rotarod and chimney test. However, Nidhi et al. [19] observed the inhibition of tolerance to DZ anticonvulsant effect by L-arginine, a donor of NO.

The involvement of NO system in tolerance phenomenon and the effects of NOS inhibitors on adaptive mechanisms related to tolerance to many drugs was the subject of numerous studies

[3, 18, 28, 32]. It was demonstrated that NOS inhibitors diminished symptoms of morphine abstinence and development of tolerance to the analgesic effects of opioids [18, 28]. Moreover, NOS inhibition by nonselective inhibitors NOS**,** L-NAME and L-NOARG, as well as by the selective inhibitor NOS, 7-NI, blocked the development of tolerance to the psychomotor impairment following ethanol administration [32]. Additionally, several reports indicated that decreased activity of NOS prevented sensitization (adaptive mechanisms associated with drug dependence) to the locomotor effect produced by CNS stimulants [28]. It is known that NO formation in the CNS is stimulated by glutamate activation of the NMDA receptors [7] and it is also supposed that the compensatory increase in glutamatergic neurotransmission is involved in BZ tolerance [2, 22]. These facts may explain, at least in part, the reducing effect of NOS inhibitors on the expression of the tolerance to the DZ-induced motor impairment in mice, which was observed in our present study.

In conclusion, the present experiments were designed to further investigate the role of NO in the adaptive changes underlying the BZ tolerance. Our results showed that nonselective NOS inhibitors attenuated the expression of the tolerance to the DZ-induced motor impairment in mice, both in the rotarod and chimney test. Thus, we can suggest that NO, at least in part, play a role in the mechanisms of BZ tolerance.

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SUMMARY

The effect of nonselective NOS inhibitors: N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME) and a NO precursor, L-arginine on the expression of tolerance to the diazepam (DZ)-induced motor impairment was investigated in this study. The DZ-induced incoordination was assessed using the rotarod and chimney tests, on the 1st and 10th days of the experiment. L-NOARG and L-NAME were found to attenuate the expression of tolerance to the motor impairing effect of DZ. The lack of influence of L-arginine on the expression of tolerance to the DZ-induced motor impairment was observed in the present experiments. These results suggest that NO may be involved, at least in part, in the tolerance to the motor dysfunction induced by BZ.

STRESZCZENIE

Celem pracy było określenie wpływu nieselektywnych inhibitorów syntazy tlenku azotu (NOS): N^G-nitro-L-argininY (L-NOARG) i estru metylowego N^G-nitro-L-argininy (L-NAME) oraz prekursora syntazy tlenku azotu (NO), L-argininy na ekspresję tolerancji na zaburzające koordynację efekty diazepamu (DZ). Koordynację ruchową zwierząt oceniano w testach pręta obrotowego i komina pierwszego i dziesiątego dnia doświadczenia. Uzyskane w pracy wyniki wykazały, iż L-NOARG i L-NAME hamują ekspresję tolerancji na badane efekty DZ. Natomiast L-arginina pozostaje bez wpływu na taką ekspresję na działanie zaburzające koordynację DZ. Uzyskane wyniki sugerują przynajmniej częściowy udział NO w tolerancji na działanie zaburzające koordynację ruchową benzodiazepin.