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Effects of sildenafil treatment on the development of tolerance to diazepam-induced motor impairment and sedation in mice

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Abstract:
We studied the effects of sildenafil, a selective inhibitor of PDE5, on the development and the expression of tolerance to diazepam (DZ)-induced motor impairment and sedation in mice. DZ-induced motor incoordination was assessed by the rotarod and chimney tests, and DZ-induced sedation was examined using a photocell apparatus. Sildenafil treatment enhanced the development of tolerance to the motor impairing effects, but not to the sedative effects, of DZ. Sildenafil treatment did not affect the expression of tolerance to DZ-induced motor impairment and sedation in mice. Our results suggest that sildenafil treatment, at least in part, affects the development of DZ tolerance.

Key words:
sildenafil, diazepam, tolerance, motor impairment, sedation, mice

Introduction

Benzodiazepines (BZs) are used to treat numerous conditions, including anxiety, insomnia, convulsive disorders, sedation and muscle relaxation. However, BZs are also classified as drugs of abuse [4, 44]. BZs act on γ-aminobutyric acid $(GABA)_A$ receptors to enhance GABA-mediated neuronal inhibition [24, 37]. BZ dependence is characterized by withdrawal upon treatment cessation and tolerance to the pharmacological effects of these drugs, such as sedation, muscle relaxation, anxiolytic effects and anticonvulsant effects. The mechanism by which BZ tolerance is conferred is unknown [1, 4]. Most authors reject the pharmacodynamic hypothesis of BZ tolerance, which is based on adaptation of the $GABA_A$ receptor. Gallager et al. [13] demonstrated that GABA responses were reduced following chronic BZ treatment through the coupling of BZ with GABA-binding sites on the $GABA_A$ receptor. In addition, it is thought that excitatory mechanisms (a likely candidate is the glutamatergic system) are sensitized to compensate for BZ-induced chronic enhancement of GABAergic inhibition [1, 31].

Nitric oxide (NO) is an important bioregulatory molecule in the nervous, immune and cardiovascular systems. It is synthesized from L-arginine, which is converted to L-citrulline by nitric oxide synthase (NOS) in the presence of O_2, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and tetrahydrobiopterin [7, 8]. There are four members of the NOS family: neuronal (nNOS), endothelial (eNOS),

inducible (iNOS) and mitochondrial NOS (mtNOS). nNOS and eNOS are Ca^{2+}-calmodulin-dependent and are constitutively expressed in mammalian cells. These cells generate transient expression of NO. In contrast, iNOS is a Ca^{2+}-calmodulin-independent enzyme, and its regulation depends on *de novo* synthesis [16]. NO binds to soluble guanylyl cyclase (sGC), a heme-containing enzyme, and thus activates cellular signaling cascades. NO allosterically interacts with sGC to increase cyclic guanosine 3',5'-monophosphate (cGMP) expression and cGMP-dependent signaling [7, 17]. cGMP, in turn, modulates the activity of cGMP-dependent kinases, cGMP-gated ion channels and cGMP-regulated phosphodiesterases (PDE) [7].

NO affects many brain functions, such as nociception, learning and memory, anxiety, seizure activity, feeding, drinking [9, 40] and the regulation of release and uptake of neurotransmitters, such as dopamine, GABA, serotonin and glutamate [9]. Moreover, NO mediates drug tolerance and dependence [2, 3, 40].

Previous studies have uncovered a relationship between L-arginine:NO:cGMP signaling and GABA-mediated transmission in the CNS. Numerous *in vivo* and *in vitro* studies suggest that NO modulates either the release or uptake of neurotransmitters, including glutamate [15] and GABA [29]. In addition, NO co-localizes with GABA [42]. It has been hypothesized that NO modulates $GABA_A$ receptor function *via* cGMP synthesis [29, 46]. Some studies demonstrate that NOS inhibition prolongs BZ-dependent sedation [33] and the anticonvulsant [34], antinociceptive [35] and anxiolytic [27] effects of BZ. Moreover, L-arginine:NO:cGMP signaling modulates tolerance to diazepam (DZ, one of the most clinically useful agents of BZ)-induced motor impairment in mice [36].

Sildenafil is commonly used to treat male erectile dysfunction [14]; however, it also affects the CNS [39]. Some studies indicate that sildenafil treatment influences neurogenesis, memory enhancement, neuroprotection and drug dependence [39]. Sildenafil is a PDE5 inhibitor that enhances the effects of NO by inhibiting cGMP degradation [14, 18, 28, 32]. Tahsili-Fahadan et al. [32] demonstrated that sildenafil has rewarding effects, which can be blocked by treatment with L-NAME (NOS inhibitor) and methylene blue (sGC inhibitor). Moreover, sildenafil has antinociceptive effects, which are potentiated by the NO donors, sodium nitroprusside and L-arginine [18].

The aim of the present study was to assess the role of sildenafil, a selective inhibitor of PDE5, in the development of tolerance to DZ-induced motor impairment and sedation. DZ is a prototypical BZ and is commonly used in the clinic [4].

Materials and Methods

Animals

We used male albino Swiss mice that had an initial weight of 20–25 g. Animals were housed in groups of ten and maintained in a 12 h light-dark cycle at a controlled temperature (21°C). They received standard food (Bacutil, Motycz, Poland) and tap water *ad libitum*. All behavioral experiments were performed 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). The protocols were also approved by the Local Ethics Committee (39/2006). A total of 240 mice were used.

Drugs

Sildenafil citrate (Fako IIaAlari A.Ş, Istanbul, Turkey) was dissolved in 0.9% saline. Diazepam (Relanium, Polfa, Poland) was diluted in 0.9% saline. Control animals were injected with the corresponding vehicle. The doses used were selected according to the literature [5, 18, 30, 32, 41].

EXPERIMENTAL PROCEDURES

Experiment 1

a) The development of tolerance to DZ-induced motor impairment in mice

Mice were administered DZ (5 mg/kg) for 10 consecutive days. Motor coordination was measured on the 1st and 10^{th} day of the experiment using the rotarod and chimney tests [21, 25, 30].

b) The effects of sildenafil (5 or 10 mg/kg) chronic administration on the development of tolerance to DZ-induced motor impairment in mice

Mice were treated with one of the following for 10 consecutive days: DZ (5 mg)/sildenafil (5 mg) and DZ (5 mg)/sildenafil (10 mg). Sildenafil was injected

30 min before DZ [5]. Experiments were initiated 30 min after the DZ injection.

To evaluate the effects of chronic sildenafil treatment on motor coordination, mice were administered sildenafil (5 or 10 mg/kg) for 10 consecutive days. The experiment was performed on day 10 after the morning sildenafil injection.

c) The effects of an acute injection of sildenafil on tolerance to DZ-induced motor impairment in mice

Mice were administered DZ (5 mg/kg) for 10 consecutive days to induce behavioral tolerance. On the 10th day of experiment, mice were administered sildenafil (5 or 10 mg/kg, *ip*) 30 min before injection of DZ (5 mg/kg, *sc*).

Experiment 2

a) The development of tolerance to DZ-induced sedation in mice

Mice were administered DZ (10 mg/kg – the morning dose and 5 mg/kg – the evening dose) or saline twice a day for 8 consecutive days. On the morning of day 9, mice were administered DZ (10 mg/kg) according to the modified method of van Rijnsoever et al. [41]. The sedative effects of diazepam were evaluated on day 1 and 9 using individual circular alleys for motor activity assessment.

b) The effects of chronic administration of sildenafil (5 or 10 mg/kg) on the development of tolerance to DZ-induced sedation in mice

Mice were treated with one of the following for 8 consecutive days: DZ (10 or 5 mg)/sildenafil (5 mg) and DZ (10 or 5 mg)/sildenafil (10 mg). Sildenafil was injected 30 min before DZ. On the morning of the test day (9th day), mice were administered sildenafil (5 or 10 mg/kg) and DZ (10 mg/kg). The experiment was initiated 30 min after the DZ injection.

To evaluate the effects of chronic sildenafil treatment on locomotor activity, mice were administered sildenafil (5 or 10 mg/kg) for 8 consecutive days twice a day. The experiment was performed on day 9 after the morning sildenafil injection.

c) The effects of acute sildenafil administration on tolerance to DZ-induced sedation in mice

Mice were administered DZ (10 mg/kg – the morning dose and 5 mg/kg – the evening dose) twice a day for 8 consecutive days to induce behavioral tolerance. On the morning of day 9, mice were administered sildenafil (5 or 10 mg/kg) 30 min before administration of DZ (10 mg/kg). The experiment was initiated 30 min after the DZ injection.

Behavioral tests

Rotarod test

Mice were trained daily for 3 days on a bar (2 cm in diameter) rotating at a constant speed of 18 rpm. During each training session, mice were placed on a rotating rod for 3 min with an unlimited number of trials. Experimentation was conducted at least 24 h after the final training trial. During the test, mice remained on the rod for as long as possible. The length of time the animal remained on the rod was recorded (a 60 s maximal trial was used for the test) [43].

Chimney test

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 [43]. Pretreatment times were 60 min for sildenafil and 30 min for diazepam.

The rotarod and chimney tests were used to evaluate the ability of drug treatment to interfere with motor coordination. However, the chimney test is also used to assess the effects of drug treatment on muscle relaxation [43].

Locomotor activity

The locomotor activity of individual mice was recorded using a photocell apparatus (round Plexiglas cage, 32 cm in diameter, Multiserv, Lublin, Poland). Animals were placed into individual cages, 60 min and 30 min after injection of sildenafil and diazepam, respectively. Cages were equipped with one row of infrared light-sensitive photocells (2 emitters and 2 sensors) located 1 cm above the floor. Locomotor activity was assessed as the number of photocell interruptions during a period of 30 min. The test was performed on day 1 and 9 of the experiment [41, 43].

Sedation, as defined by Katzung [19], is assessed as a drug-induced decrease in the animal's spontaneous activity. The measurement of motor activity is

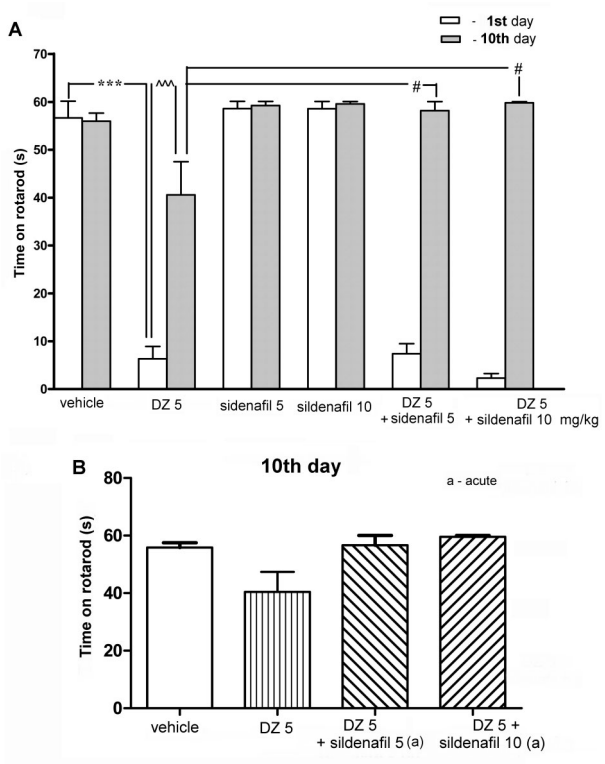

Fig. 1. The influence of sildenafil treatment (5 or 10 mg/kg, *ip*) on the development (**A**) and the expression (**B**) of tolerance to diazepam-induced motor impairment (DZ, 5 mg/kg, *sc*) as assessed by the rotarod test. Data represent the mean ± SEM of a group of 10 mice (*** $p < 0.001$ *vs.* vehicle; ^^^ $p < 0.001$ *vs.* DZ 5 (1st day); # $p < 0.05$ *vs.* DZ 5 (the test day) (Tukey-Kramer's test))

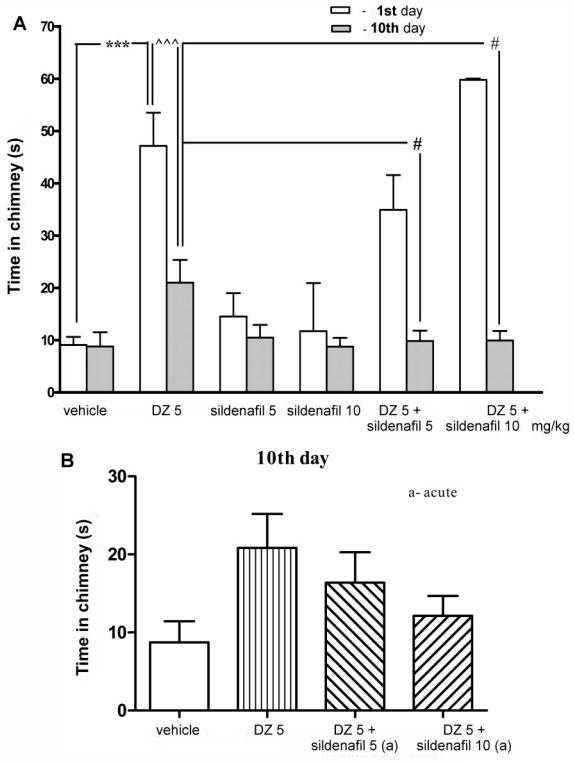

Fig. 2. The influence of sildenafil treatment (5 or 10 mg/kg, *ip*) on the development (**A**) and the expression (**B**) of tolerance to diazepam-induced motor impairment (DZ, 5 mg/kg, *sc*) as assessed by the chimney test. Data represent the mean ± SEM of a group of 10 mice (*** $p < 0.001$ *vs.* vehicle; ^^^ $p < 0.001$ *vs.* DZ 5 (1st day); # $p < 0.05$ *vs.* DZ 5 (the test day) (Tukey-Kramer's test))

a standard behavioral assay for testing the sedative effects of a drug [43].

Statistical analysis

Data were analyzed by one-way ANOVA. *Post-hoc* comparisons were performed using the Tukey-Kramer test. A p value less than 0.05 was considered statistically significant. Data are presented as the mean ± SEM.

Results

Effects of DZ on performance in the rotarod test (Fig. 1A, 1B) and chimney test (Fig. 2A, 2B)

Acute administration of DZ (5 mg/kg) on day 1 impaired mouse motor coordination. This effect was observed both in the rotarod test ($p < 0.001$) and chimney test ($p < 0.001$). Chronic (10 days) treatment with DZ (5 mg/kg/day) resulted in the development of tolerance to its motor impairing effects, which was observed both in the rotarod and chimney test. The results of the acute (day 1) and chronic DZ (day 10) treatments were significantly different.

The influence of sildenafil treatment on the development of tolerance to DZ-induced motor impairment as assessed by the rotarod test (Fig. 1A) and chimney test (Fig. 2A)

Sildenafil (5 or 10 mg/kg/day) co-administered with DZ facilitated the development of tolerance to the motor impairment effects of DZ as assessed by the rotarod ($p < 0.05$) and chimney test ($p < 0.05$) on day 10 of the experiment. Acute and chronic (10 days) sildenafil (5 or 10 mg/kg) treatment did not significantly affect motor coordination as measured by the rotarod and chimney tests.

The influence of sildenafil treatment on the expression of tolerance to DZ-induced motor impairment as assessed by the rotarod (Fig. 1B) and chimney test (Fig. 2B)

Co-administration of an acute injection of sildenafil (5 or 10 mg/kg; day 10) after chronic treatment with DZ (5 mg/kg, 10 days) did not affect the expression of tolerance to DZ-induced motor impairment as measured by the rotarod and chimney test.

Effects of DZ treatment on mouse performance in the photocell apparatus (Fig. 3A, 3B)

Acute administration of DZ (10 mg/kg) on day 1 caused sedation in mice as measured in a photocell apparatus ($p < 0.001$). Chronic treatment (9 days) with DZ (10 + 5 mg/kg/day; on day 9 – only 10 mg/kg) resulted in the development of tolerance to its sedative effect, which was observed as an increase in locomotor activity in a photocell apparatus. The effects of the acute (day 1) and chronic DZ treatments (day 9) ($p < 0.05$) were significantly different.

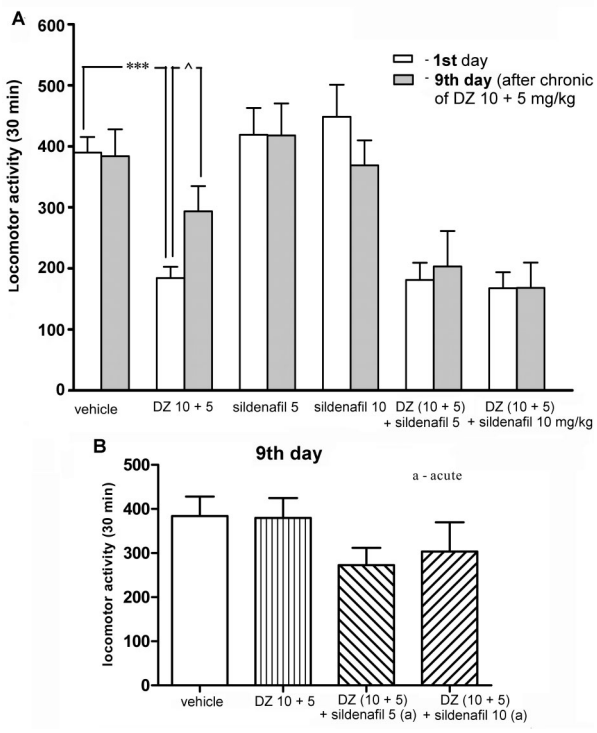

Fig. 3. The influence of sildenafil treatment (5 or 10 mg/kg, *ip*) on the development (**A**) and the expression (**B**) of tolerance to diazepam-induced sedation (DZ, 10 + 5 mg/kg, *sc*) as assessed in a photocell apparatus. Data represent the mean ± SEM of a group of 10 mice (*** $p < 0.001$ *vs.* vehicle; ^ $p < 0.05$ *vs.* DZ 5 (1st day))

The influence of sildenafil treatment on the development of tolerance to the sedative effects of DZ as measured in a photocell apparatus (Fig. 3A)

Chronic co-administration of sildenafil (5 or 10 mg/kg) with DZ (10 + 5 mg/kg) did not affect the development of tolerance to the sedative effects of DZ; there was no difference in the number of photocell interruptions on day 9 compared to day 1 of the experiment. Acute and chronic (9 days) treatment with sildenafil (5 or 10 mg/kg) did not significantly affect locomotor activity as assessed by in the photocell apparatus.

The influence of sildenafil treatment on the expression of tolerance to the sedative effect of DZ as measured in a photocell apparatus (Fig. 3B)

Acute injection of sildenafil (5 or 10 mg/kg; day 9) during chronic treatment with DZ (10 or 5 mg/kg; 8 days; on day 9 – only 10 mg/kg) did not affect tolerance to the sedative effect of DZ as assessed by the number of photocell interruptions on day 9 of the experiment.

Discussion

Mice developed tolerance to DZ-induced motor impairment and sedation after chronic treatment. Tolerance to some effects of BZ (i.e., sedative effects, motor disturbances or anxiolytic effects) has been demonstrated in many animal studies [10–12]. However, this attenuation of the drug-induced effects does not occur for every effect simultaneously (e.g., tolerance to the sedative effect and to loss of motor coordination develops more rapidly than tolerance to the anticonvulsant effect) [4, 6, 12]. This characteristic suggests that different mechanisms are responsible for the development of tolerance to each of these behavioral parameters and/or different brain regions and BZ receptor subunits are involved in these mechanisms [4, 41, 44]. It has been suggested that the loss of BZ therapeutic efficacy after a chronic treatment results from adaptive processes that counteract BZ-enhanced $GABA_A$ receptor activity [1, 10, 12]. In addition, there is evidence of BZ receptor down-regulation [38], alteration in the expression of $GABA_A$ receptor

subunits [44] and functional allosteric uncoupling of the BZ receptor recognition site on the $GABA_A$ receptor [13]. However, these adaptive processes may not be the only mechanisms involved in the development of BZ tolerance. A theoretical model of drug tolerance postulates that pharmacodynamic tolerance develops as a result of an increase in compensatory mechanisms (an increase in excitatory glutamatergic neurotransmission) [1, 6].

Importantly, our study demonstrates that treatment with sildenafil (5 or 10 mg/kg), a potent, reversible and selective inhibitor of PDE5, enhanced the development of tolerance to the motor impairing effects of DZ as assessed in the rotarod and chimney test. Our study also showed that acute or chronic administration of sildenafil did not affect motor coordination in mice. Interestingly, our previous study demonstrated that chronic pre-treatment with L-arginine, an endogenous NO precursor, enhanced tolerance to DZ-induced motor impairment as assessed by the rotarod test [36].

However, sildenafil treatment (5 or 10 mg/kg) did not affect the development of tolerance to the sedative effect of DZ. Neither acute nor chronic sildenafil treatment induced sedation at each tested dose.

Moreover, our data demonstrate that acute administration of sildenafil, (both 5 and 10 mg/kg) to animals chronically injected with DZ did not affect tolerance to DZ-induced motor impairment and sedation. This result suggests that the development of tolerance is associated with various complex adaptive mechanisms [4, 40].

A wealth of data support the hypothesis that NO may be involved in the development of tolerance to substances of abuse, such as opioids, ethanol, nicotine and psycho-stimulants [3, 20, 22, 23, 40, 45]. Treatment with NOS inhibitors diminishes the symptoms of morphine abstinence and the development of tolerance to the analgesic effects of opioids [23, 40]. Moreover, NOS inhibition by nonselective inhibitors, NOS, N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NOARG), as well as by the selective inhibitor of NOS, 7-nitroindazole (7-NI), blocks the development of tolerance to ethanol-induced psychomotor impairment [45]. In addition, several reports indicate that decreased NOS activity prevents sensitization (adaptive mechanisms associated with drug dependence) to the locomotor effects of CNS stimulants [40].

Interestingly, some data associate the NO system with tolerance to BZ treatment, yet results of these studies are not clear. Nidhi et al. [25] recently reported that L-NOARG treatment did not prevent the development of tolerance to the anticonvulsant effects of DZ in rats. In addition, they determined that treatment with L-arginine, a NO donor, did not diminish tolerance to DZ anticonvulsant effects. In contrast, our previous studies showed that treatment with L-NAME, L-NOARG and 7-NI prevents the development of tolerance to the motor impairing effects of DZ, whereas L-arginine treatment facilitates this tolerance as assessed by the rotarod test [36]. Such conflicting data suggest that the tolerance to different effects of BZ is mediated by various mechanisms.

The mechanisms by which the modification of the L-arginine:NO:cGMP pathway affects BZ tolerance are not fully understood. It is suggested that chronic treatment with sildenafil and diazepam prevents $GABA_A$ receptor down-regulation and/or glutamate receptor up-regulation during the development of tolerance to the motor impairing effects of diazepam. However, tolerance to the sedative effects of BZ correlates with a decrease in glutamate sensitivity and an increase in hippocampal synaptic plasticity [6]. In our study, sildenafil treatment did not affect the development of tolerance to the sedative effects of DZ, suggesting that different mechanisms elicit this type of BZ tolerance. The development of BZ tolerance may be directly related to the diversity of $GABA_A$ receptor subtypes. Moreover, it should be noted that sildenafil treatment inhibits PDE5 and activates NO in the CNS; however, it can also act through other mechanisms (e.g., it has been demonstrated that sildenafil is linked with the dopaminergic system) [26]. Further study concerning sildenafil and its influence on the development of BZ tolerance is necessary.

In conclusion, our data indicate that treatment with sildenafil, a selective inhibitor of PDE5, facilitates the development, but not expression, of tolerance to the motor impairing effects of DZ. The lack of effect of sildenafil treatment on the development and expression of tolerance to sedation in mice was observed. These data suggest that sildenafil treatment, at least in part, affects the development of BZ tolerance.

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