ELSEVIER

European Journal of Pharmacology 351 (1998) 287–290

ejp

Short communication

L-Arginine abolishes the anxiolytic-like effect of diazepam in the elevated plus-maze test in rats

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Received 6 May 1998; accepted 12 May 1998

Abstract

The involvement of nitrergic mechanisms in the behavioural effects of diazepam in rats was studied in the elevated plus-maze, open-field and rotarod tests. Administration of the nitric oxide (NO) precursor L-arginine (100 mg/kg, i.p.), assumed to increase the synthesis of NO, abolished the anxiolytic-like effect of diazepam (2 mg/kg, i.p.) in the elevated plus-maze, whereas the inactive enantiomer D-arginine (100 mg/kg) did not. Neither diazepam alone nor in combination with L- or D-arginine affected the exploratory activity of animals in the open field. Pretreatment with L-arginine (100 and 200 mg/kg) did not modify the motor impairment of rats after diazepam (3 mg/kg) in the rotarod test. Diazepam (2 mg/kg i.p.) did not inhibit the cortical or hippocampal cytosolic NO synthase activity measured ex vivo by [^{3}H]L-arginine assay. Diazepam was similarly ineffective in in vitro studies at concentrations up to 10 μM. We conclude that a suppression of NO synthase activity may be important in the anxiolytic-like effect of benzodiazepines. However, diazepam does not inhibit NO synthase directly, but may affect NO synthase activity indirectly via some unknown mechanism.

Keywords: Diazepam; Nitric oxide (NO) synthase; L-Arginine; Anxiety; Motor performance; (Rat)

1. Introduction

Nitric oxide (NO) has been recognized as an intercellular messenger in the central nervous system (Bredt and Snyder, 1989). Interestingly, NO synthase inhibitors share several pharmacological properties with benzodiazepine agonists, e.g., they have an anxiolytic-like effect, decrease motor activity, possess an anticonvulsant effect and decrease cyclic GMP content (Volke et al., 1997; Faria et al., 1997; Mülsch et al., 1994; Bredt and Snyder, 1989; Ongini et al., 1982). Therefore, we hypothesized that the brain nitrergic system may be involved in some of the effects of benzodiazepines. The aim of the present study was to elucidate whether the behavioural effects of a reference benzodiazepine anxiolytic, diazepam, could be modulated by the NO precursor L-arginine. Moreover, the effect of diazepam on the brain NO synthase activity was measured in vitro and ex vivo.

2. Materials and methods

2.1. Animals

Male Wistar rats (The National Animal Center, Kuopio, Finland) weighing 200–250 g were used. Rats were housed in cages of four at 20 ± 2°C in a 12-h light/dark cycle (light on at 0700 h). Tap water and food pellets were available ad libitum.

2.2. Elevated plus-maze test

The apparatus and procedure have been previously described (Volke et al., 1997). During a 5-min observation period, the following parameters were measured: number of open arm entries, time spent on open arms and number of closed arm entries. Subsequently, the percentage of the number of entries into the open arms of the total number of entries into all arms and percentage of time spent on open arms were calculated.

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0014-2999/98/$19.00

2.3. Open field test

Immediately after the plus-maze test, the rats were placed singly into an open field (1 m × 1 m, divided by lines into 16 equal squares) and observed in 4 min to measure locomotor activity. The number of line crossings and rearing were counted.

2.4. Rotarod

The rotarod performance was measured as described by Suzdak et al. (1992). The rats were trained on the day before the test to stay on the rotating wooden bar (diameter 8 cm, speed 9 rpm) for at least 3 min. On the test day, the animals were put on the rotarod and the number of falls during a 3-min session was registered.

2.5. NO synthase assay

Brain pieces (frontal cortex and hippocampus) were homogenized (1:10 w/v) in ice-cold 20-mM Tris–HCl buffer (pH 7.4) containing 2 mM of EDTA. After centrifugation (20 000 × g, 15 min) supernatants were removed and used immediately to measure NO synthase activity. An aliquot of supernatant (25 μl) was added to the reaction buffer (40-mM HEPES containing 1 mM of $CaCl_2$, 1 mM of NADPH and 120 nM of L-[2,3,4,5-^{3}H]L-arginine, pH 7.4), final volume 100 μl, and incubated 30 min at 37°C. For in vitro experiments the aliquots (20 μl) were preincubated 15 min with 10 μl of diazepam on ice, followed by addition of 70 μl of reaction buffer and incubation (15 min at 37°C). The blank samples received buffer without $CaCl_2$ and NADPH. The reaction was stopped by addition of 1 ml of ice-cold 20-mM HEPES buffer containing 2 mM of EDTA and subsequent transfer to ice. [^{3}H]L-citrulline was separated using 0.5-ml columns of Dowex AG50WX-8 and quantified by the liquid scintillation spectroscopy. Protein concentrations were measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.6. Drugs

7-Nitroindazole was obtained from RBI (Natick, USA). L-[2,3,4,5-^{3}H]Arginine was purchased from Amersham (Amersham, UK). All other chemicals were from Sigma (St. Louis, USA). L- and D-arginine were dissolved in saline. 7-Nitroindazole and diazepam were dissolved using a few drops of Tween-80 for in vivo experiments and dimethylsulphoxide, final concentration 10%, for in vitro experiments. All drugs were freshly prepared and given intraperitoneally (i.p.) in a volume of 0.1 ml/100 g body weight of rats at different time intervals before the testing.

2.7. Statistics

Data were statistically treated using one-way analysis of variance (ANOVA). Post-hoc comparisons between individual groups were performed by Duncan's multiple range test. Data are expressed as the mean values ± S.E.M.

3. Results

3.1. Elevated-plus-maze and open field

The results are shown in Fig. 1. One-way ANOVA indicated significant drug effects on percentage of time spent on, percentage of entries into open arms and total number of arm entries ($F = 3.75$, $P < 0.05$; $F = 8.12$, $P < 0.001$ and $F = 3.81$, $P < 0.05$, respectively). Diazepam (2 mg/kg i.p.) caused a significant increase in percentage of open arm visits ($P < 0.05$), in total number

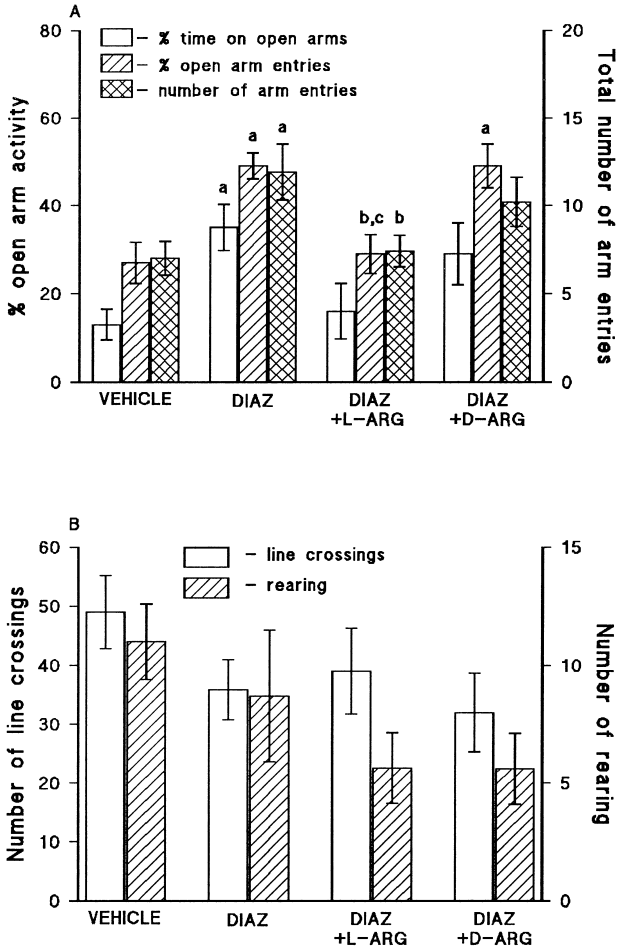

Fig. 1. The effect of pretreatment with L- or D-arginine (100 mg/kg i.p. 60 min prior to the test) on the behavioural action of diazepam (2 mg/kg, i.p. 30 min prior the test) in the elevated plus-maze. (A, test duration 5 min) and open field (B). $n = 8–10$. (a) $P < 0.05$ compared to control group, (b) $P < 0.05$ compared to diazepam group, (c) $P < 0.05$ compared to diazepam + D-arginine group, Duncan's test after the significant one-way ANOVA.

of arm entries ($P < 0.05$) and in percentage of time spent on open arms ($P < 0.05$). Pretreatment with L-arginine (100 mg/kg i.p. 60 min before testing), but not with D-arginine, abolished the anxiolytic-like effect of diazepam ($P < 0.05$; Fig. 1A). ANOVA did not show any drug effect on the number of line crossings ($F = 1.25$, $P > 0.1$) or number of rearing ($F = 1.72$, $P > 0.1$). However, a post-hoc test indicated that the decrease in number of rearing was close to statistical significance in the case of diazepam combined with L- or D-arginine ($P = 0.052$ and 0.06 respectively) compared to the vehicle group (Fig. 1B).

3.2. Rotarod performance

Treatment had significant effect on the motor coordination of rats on rotarod ($F = 6.0$, $P < 0.01$). Diazepam (3 mg/kg) increased the number of falls during the 3-min test session from 0.4 ± 0.2 to 9.4 ± 2.4 ($P < 0.01$). Pretreatment with L-arginine (100 or 200 mg/kg) did not modify the motor incoordination caused by diazepam (number of falls 8.4 ± 1.9 and 9.6 ± 1.9 respectively).

3.3. NO synthase activity

ANOVA revealed a significant drug effect on the cortical and hippocampal NO synthase measured ex vivo ($F = 60$, $P < 0.0001$ and $F = 46$, $P < 0.0001$, respectively). The NO synthase inhibitor, 7-nitroindazole (40 mg/kg i.p. 40 min prior sampling) decreased NO synthase activity by more than 65% ($P < 0.001$) in cortex and 70% ($P < 0.001$) in hippocampus, as shown in Fig. 2A. Diazepam (2 mg/kg i.p. 30 min prior sampling) did not affect NO synthase activity. Diazepam had no apparent effect on hippocampal NO synthase activity in vitro in concentrations up to 10 μM (Fig. 2B).

4. Discussion

The main finding of the present study is that pretreatment with L-arginine abolished the anxiolytic-like effect of diazepam in the elevated plus-maze. L-arginine seems to cause a true change in the anxiolytic-like action of diazepam since Faria et al. (1997) as well as our group (Volke et al., 1997) have shown that L-arginine, (60 and 100 mg/kg i.p., respectively) administered alone, does not modify the behaviour of rats in the elevated plus-maze test. Moreover, the effect of the L-arginine treatment to decrease entries into open arms of the maze could not be explained by nonspecific change in motor activity, since L-arginine combined with diazepam did not affect locomotion of rats in the open field test. Since only the NO precursor L-arginine, but not the biologically inactive enantiomer D-arginine, modified the behaviour of the rats, the increased synthesis of NO seems likely to account for the above-mentioned action. L-arginine (150 and 300 mg/kg i.p.) has been shown to increase brain NO synthesis by about 50% (Salter et al., 1996). The present results are in line with the previous findings showing that specific NO synthase inhibitors induce an anxiolytic-like effect in the elevated plus-maze (Wiley et al., 1995; Volke et al., 1997; Faria et al., 1997) and indicating that NO may be an anxiogenic stimulus. However, not all the evidence is unanimous. Quock and Nguyen (1992) have described that the NO synthase inhibitor, L-NG-nitro arginine (L-NOARG) antagonizes the anxiolytic-like effect of chlordiazepoxide. Moreover, De Oliveira et al. (1997) showed that L-NOARG has an anxiogenic-like effect in the elevated plus-maze.

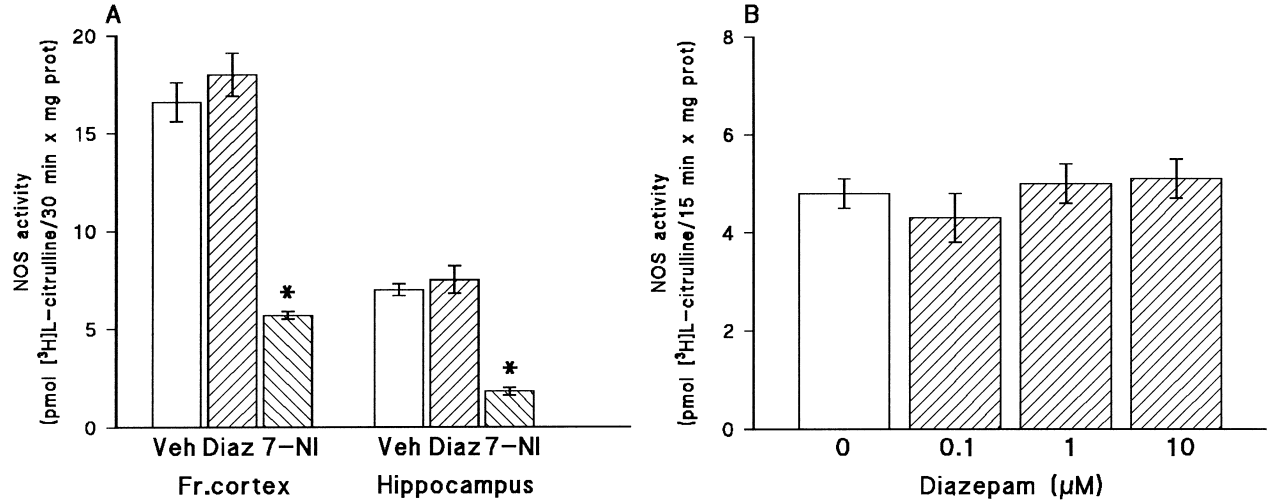

Fig. 2. (A) The action of 7-nitroindazole (40 mg/kg i.p.) and diazepam (2 mg/kg) on brain NO synthase activity ex vivo. (B) The effect of diazepam on hippocampal NO synthase activity in vitro. $n = 5$, $* P < 0.001$, Duncan's test after the significant one-way ANOVA.

We do not have any explanation for these discrepancies, but non-specific effects of the drug on locomotion cannot be ruled out.

We are unable to assess whether an increase in NO synthesis affects the benzodiazepine induced hypolocomotion, because the dose of diazepam used (2 mg/kg) did not significantly suppress the motor activity. L-arginine did not modify the motor impairment caused by diazepam (3 mg/kg), even though the dose of L-arginine was increased to 200 mg/kg, demonstrating that not all the effects of diazepam are sensitive to an increase of the NO synthesis.

In another set of experiments we tested the hypothesis that diazepam may directly affect the NO synthase activity, which in turn would explain the behavioural effect of L-arginine. However, diazepam did not modify NO synthase activity as measured ex vivo, whereas the known NO synthase inhibitor 7-nitroindazole (40 mg/kg) decreased activity by more than 60%. Diazepam (in concentrations up to 10 μM) was similarly ineffective in in vitro studies, demonstrating that there is no direct interaction of diazepam with the NO synthase. Nevertheless, it is tempting to speculate that benzodiazepines may decrease NO synthase activity via some indirect mechanism, e.g., by changing the concentration of some essential cofactor. Indeed, Wang et al. (1997) have found that γ-aminobutyric acid (GABA) positive neurons have synapses to NO synthase positive neurons in the dorsal raphe nucleus, suggesting that GABAergic neurons could modulate NO-producing neurons. Furthermore, Mülsch et al. (1994) showed that diazepam (10 mg/kg) is nearly as effective in inhibiting the kainate-induced NO formation as the NO synthase inhibitor, 7-nitroindazole. There are also other potential levels of interaction between GABA- and nitrergic systems in the regulation of anxiety. An increased synthesis of NO can decrease GABA-stimulated Cl^- influx by inhibiting the function of the GABAA receptor as described by Zarri et al. (1994). In fact, the functional balance between GABAergic and nitrergic system may be important in the regulation of anxiety but further studies are needed to clarify the impact and mechanism of this interaction. In conclusion, the present study indicates that the anxiolytic-like effect of diazepam depends on the level of NO synthesis. However, diazepam is not an inhibitor of NO synthase, but may affect the NO synthase balance indirectly via some unknown mechanism.

Acknowledgements

This study was partly granted by the Estonian Science Foundation (grant No. 2980). V. Volke and S. Kõks held a scholarship from Center of International Mobility (CIMO), Finland.

References

Bredt, D.S., Snyder, S.H., 1989. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. USA 86, 9030–9033.

De Oliveira, C.L., Del Bel, E.A., Guimaraes, F.S., 1997. Effects of L-NOARG on plus-maze performance in rats. Pharmacol. Biochem. Behav. 56, 55–59.

Faria, M.S., Muscara, M.N., Moreno, H. Jr., Teixeira, S.A., Dias, H.B., De Oliveira, B., Graeff, F.G., De Nucci, G., 1997. Acute inhibition of nitric oxide synthesis induces anxiolysis in the plus-maze test. Eur. J. Pharmacol. 323, 37–43.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mülsch, A., Busse, R., Mordvintcev, P.I., Vanin, A.F., Nielsen, E.O., Scheel-Krüger, J., Olesen, S.P., 1994. Nitric oxide promotes seizure activity in kainate-treated rats. NeuroReport 5, 2325–2328.

Ongini, E., Iuliano, E., Racagni, G., 1982. Cerebellar cyclic GMP and behavioral effects after acute and repeated administration of benzodiazepines in mice. Eur. J. Pharmacol. 80, 185–190.

Quock, R.M., Nguyen, E., 1992. Possible involvement of nitric oxide in chlordiazepoxide-induced anxiolysis in mice. Life Sci. 51, 255–260.

Salter, M., Duffy, C., Garthwaite, J., Strijbos, P.J.L.M., 1996. Ex vivo measurement of brain tissue nitrite and nitrate accurately reflects nitric oxide synthase activity in vivo. J. Neurochem. 66, 1683–1690.

Suzdak, P.D., Frederiksen, K., Andersen, K.E., Sørensen, P.O., Knutsen, L.J.S., Nielsen, E.B., 1992. NNC-711, a novel potent and selective γ-aminobutyric acid uptake inhibitor: pharmacological characterization. Eur. J. Pharmacol. 223, 189–198.

Volke, V., Soosaar, A., Kõks, S., Bourin, M., Männistö, P.T., Vasar, E., 1997. 7-Nitroindazole, a nitric oxide synthase inhibitor, has anxiolytic-like properties in exploratory models of anxiety. Psychopharmacology 131, 399–405.

Wang, Q.-P., Guan, J.-L., Nakai, Y., 1997. Electron microscopic study of GABAergic synaptic innervation of nitric oxide synthase immunoreactive neurons in the dorsal raphe nucleus in the rat. Synapse 25, 24–29.

Wiley, J.L., Cristello, A.F., Balster, R.L., 1995. Effects of site-selective NMDA receptor antagonists in an elevated plus-maze model of anxiety in mice. Eur. J. Pharmacol. 294, 101–107.

Zarri, I., Bucossi, G., Cupello, A., Rapallino, M.V., Robello, M., 1994. Modulation by nitric oxide of rat brain GABAA receptors. Neurosci. Lett. 180, 239–242.