Possible Underlying Influence of p38MAPK and NF-κB in the Diminished Anti-anxiety Effect of Diazepam in Stressed Mice

Vipin Sharma1, Ritu Gilhotra1, Dinesh Dhingra2, and Neeraj Gilhotra3,*

1School of Pharmacy, Gyan Vihar University, Jaipur – 302 025, Rajasthan, India
2Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar – 125 001, Haryana, India
3Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak – 124 001, Haryana, India

Received February 9, 2011; Accepted March 24, 2011

Abstract. The present study was designed to explore the possible nitriergic influence and role of p38MAPK and NF-κB in the diminished anti-anxiety effect of diazepam in stressed mice, using the elevated plus maze and light/dark box to assess anxiety. Immobilization stress for 6 h enhanced an anxiety-like behavior and increased plasma nitrite levels in mice. Diazepam (2 mg/kg, i.p.) produced an anti-anxiety effect in unstressed mice, but could not produce any change in anxiety levels of stressed mice. SB-203580 (2 mg/kg, i.p.), a specific inhibitor of p38MAPK, per se produced a significant anti-anxiety-like activity in stressed mice. Administration of a combination of SB-203580 (2 mg/kg, i.p.) and diazepam (2 mg/kg) in stressed mice produced a significantly higher anti-anxiety-like activity than that produced by SB-203580 alone. Pyrrolidine dithiocarbamate (PDTC), per se produced a significant anti-anxiety-like activity in stressed mice. Combination of PDTC and diazepam also served to produce a higher significant anti-anxiety-like activity in stressed mice than that produced by PDTC alone. Diazepam could not produce any change in plasma nitrite levels in both unstressed and stressed mice. Both SB-203580 (2 mg/kg, i.p.) and PDTC (100 mg/kg, i.p.) significantly decreased plasma nitrite levels in stressed mice. The observations indicate that the diminished anti-anxiety effect of diazepam in stressed mice may involve strong nitriergic influence and may further be p38MAPK- and NF-κB–dependent.

Keywords: anxiety, diazepam, immobilization, pyrrolidine dithiocarbamate (PDTC), SB-203580

Introduction

Stress can influence the neurobehavioral profile and precipitate an anxiety-like syndrome (1, 2). Acute (6 h) stress activates nitric oxide synthase (NOS) and enhances anxiety in rodents (3 – 8). Stress has been demonstrated to activate p38 mitogen-activated protein kinase (p38MAPK) (9, 10). p38MAPK is a serine-threonine kinase, mediating many cellular responses to chemical and physical insults (11). Activation of p38MAPK results in the expression of inducible nitric oxide synthase (iNOS) and release of nitric oxide (NO) (12 – 14).

Acute stress (6 h) has also been demonstrated to stimulate the translocation of the nuclear factor-kappa B (NF-κB) to the nucleus after 4 h of immobilization (15). This inducible activation of NF-κB stimulates the transcription of the iNOS gene, leading to an increase in NO production (16). The administration of the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) has been shown to inhibit the immobilization stress (6 h)-induced increase in iNOS expression (15).

Immobilization stress can also disturb GABAergic receptors and benzodiazepine coupling to these receptors and induce persistent changes in the GABA-benzodiazepine–barbiturate complex in the brain of stressed animals along with sub-sensitivity of central GABA receptors (17, 18). Behavioral effects of drugs acting at the GABA–benzodiazepine–barbiturate complex may vary in stressed animals as compared to unstressed animals (19). Further, iNOS-derived NO and subsequent activa-
tion of an endogenous NO-sensitive guanylyl cyclase (GC), results in an increased levels of cyclic guanosine monophosphate (cGMP) (20, 21), which may downregulate GABA<sub>A</sub> receptor function in the hippocampus, an area involved in anxiety (22, 23). Moreover, NO analogues have been found to reduce GABA-gated currents via cGMP-dependent pathways, leading to anxiety (24). Further, it has been recently observed that 6-h immobilization reduces GABA contents in mouse brains and that the anti-anxiety effect of diazepam (2 mg/kg, i.p.) is compromised in stressed mice (8).

Therefore, the present study is designed to investigate primarily the role of p38MAPK by employing SB-203580, a selective inhibitor of p38MAPK. PDTC, an inhibitor of NF-κB, was also used in combination with diazepam to explore whether this is NF-κB–dependent process, as our recent work shows that PDTC can produce antianxiety-like activity in stressed mice (25).

**Materials and Methods**

**Animals**

Swiss albino mice (male, 20 – 25 g) were employed in the present study. Animals were procured from Disease Free Small Animal House, CCS Haryana Agricultural University, Hisar, Haryana, India. Animals were provided normal diet and tap water ad libitum and were exposed to 12-h light and 12-h dark cycle. The animals were acclimatized to the laboratory condition before experiments. Experimental protocol was approved by Institutional Animal Ethics Committee. Care of the animals was taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

**Drugs**

SB-203580 and PDTC were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diazepam (Calmpose<sup>®</sup>) was obtained as an ampoule from Ranbaxy Laboratories, Ltd., Gurgaon, India.

**Elevated plus maze (EPM)**

The plus maze apparatus consisted of two open arms, 16 × 5 cm, and two closed arms, 16 × 5 × 12 cm, connected to a central platform (5 × 5 cm). The maze was elevated to a height of 25 cm above the floor. Each mouse was placed individually at the centre of EPM with its head facing towards an open arm and observed for 5 min to record the number of entries into the open arm, closed arm, and time spent in each arm (26). In the EPM test, percent time spent on the open arms was determined as follows:

\[
\text{Percent time on open arms} = \frac{\text{Number of seconds spent on open arms}}{300} \times 100
\]

**Light and dark test (LDT)**

The apparatus consisted a rectangular box (45 × 27 × 27 cm), partitioned into two compartments connected by a 7.5 × 7.5 cm opening in the wall between compartments. An animal was placed into the center of the light compartment and was observed for 5 min for the time spent in the open (white/light) compartment (27). Percent time spent in the light compartment was determined as follows:

\[
\text{Percent time in light compartment} = \frac{\text{Number of seconds spent in light compartment}}{300} \times 100
\]

**Plasma nitrite estimation**

For nitrite estimation, blood was withdrawn from the tail vein of immobilized mice immediately before setting the animal free and subjecting it to behavioral tests in all the groups. The sampling procedure was completed during immobilization to avoid the extra stress incurred upon the mice during a new procedure of mouse immobilization for handling the tail of mice. Plasma was separated using a refrigerated centrifuge at 2500 rpm for 10 min. It was stored in a refrigerator and processed for nitrite estimation within 24 h (7, 8, 25). Plasma nitrite was measured by a spectrophotometric assay based on Griess reaction (28).

**Experimental protocol**

Experimental animal groups employed in the present study consisted of six mice each. Stress was produced in mice by immobilizing them for 6 h (8 a.m. – 2 p.m.) by taping all its four limbs and trunk on a wooden board (7, 8, 25). Mice subjected to immobilization were called stressed mice. Unstressed mice were exposed to the EPM and LDT for a normal duration (5 min), sufficient to assess the anxiety levels in rodents (29) and not subjected to immobilization and mentioned accordingly in the manuscript. Behavioral tests were performed in independent groups of mice. Vehicle, diazepam, and SB-203580 were administered in separate groups of mice 30 min before subjecting them to behavioral testing in unstressed mice. Drugs were administered 30 min before the immobilization session in case of the stressed group. The dosage schedule in the stressed group assures that the treatment(s) employed inhibited any change(s) occurring immediately after and during immobilization, thereby producing the net change in behavior or biochemical parameter of mice under investigation (6 – 8, 30).

**Locomotor activity**

The effects of various treatments on spontaneous loco-
motor activity of animals were measured by using an actophotometer (INCO, Ambala, India).

Statistical analyses
All the results are expressed as the mean ± S.E.M. Data were analyzed by analysis of variance (ANOVA) in the GraphPad Instat (GPIS) package, version 3.05 (GraphPad Software, San Diego, CA, USA); *P* < 0.05 was considered to indicate a significant difference.

Results

Anxiolytic-like activity is indicated by a significant increase in percentage of time spent in the open arms in the EPM and a significant increase in percentage of time spent in the light compartment in the LDT. On the other hand, a significant decrease in the said parameters indicate anxiogenic effect.

Effect of different treatments on mice behavior

In the EPM test, 6-h immobilization significantly decreased the percentage of time spent in the open arms by unstressed mice, as compared to that by vehicle-treated unstressed mice. In the LDT, immobilization significantly decreased the percentage of time spent in the light compartment by unstressed mice as compared to that by vehicle-treated unstressed mice (Figs. 1 and 2). Diazepam (2 mg/kg, i.p.) significantly increased percentage of time spent in the open arms of the EPM by unstressed mice, as compared to that by vehicle-treated unstressed mice. On the other hand, in stressed mice (6-h immobilized mice), diazepam (2 mg/kg, i.p.) could not produce a significant anti-anxiety effect in the EPM. In the light/dark box, diazepam (2 mg/kg, i.p.) significantly increased percentage of time spent by the unstressed mice in the light compartment. Like EPM, diazepam (2 mg/kg, i.p.) could not produce a significant anti-anxiety effect in stressed mice (6-h immobilized mice), tested in the light/dark box (Figs. 1 and 2). SB-203580 at all doses (0.5, 1, or 2 mg/kg, i.p.) used in the present study did not produce any change in anxiety levels of mice exposed to EPM and LDT for 5 min, that is, unstressed mice. On the other hand, in stressed mice (exposed to 6-h immobilization), the highest dose of SB-203580 (2 mg/kg, i.p.) employed, produced a significant antianxiety-like effect in both EPM and LDT (Figs. 1 and 2). PDTC (100 mg/kg, i.p.) did not produce any change in anxiety levels of mice, exposed to EPM and LDT for 5 min, that is, unstressed mice. On the other hand, in stressed mice (exposed to 6-h immobilization), PDTC produced a significant antianxiety-like effect in both EPM and LDT (Figs. 1 and 2). SB-203580 (2 mg/kg, i.p.) pre-treatment did not produce any change in the anti-anxiety effect of diazepam in unstressed mice in EPM and LDT. On the other hand, SB-203580 (2 mg/kg, i.p.) pretreatment served to produce a significant anti-anxiety effect in diazepam-treated stressed mice. The noted anti-anxiety effect was observed to be higher than that produced by SB-203580 and diazepam alone in stressed mice (Figs. 1 and 2). PDTC (100 mg/kg, i.p.) pre-treatment did not produce any change in the anti-anxiety effect of diazepam in unstressed mice in EPM and LDT. On the other hand, PDTC (2 mg/kg, i.p.) pretreatment served to produce a significant anti-anxiety effect in diazepam-treated stressed mice. The noted anti-

Fig. 1. Effect of different treatments on percentage of time spent by mice in open arms of elevated plus maze. Values are expressed as mean ± S.E.M. (n = 6 in each group). Data were analyzed by one-way ANOVA followed by Tukey’s post hoc test, *P* < 0.0001. a = *P* < 0.05, significant difference from vehicle-treated control group (unstressed mice); b, c, d, e = significant difference from immobilization group (stressed mice); c = significant difference from diazepam-treated stressed mice and SB-203580–treated stressed mice; e = significant difference from diazepam-treated stressed mice and PDTC-treated stressed mice. Veh: vehicle, IMMO: immobilization, DZP(U): diazepam (unstressed mice), DZP(S): diazepam (stressed mice), SB(U): SB-203580 (unstressed mice), SB(S): SB-203580 (stressed mice), PDTC(U): pyrrolidine dithiocarbamate (unstressed mice), PDTC(S): pyrrolidine dithiocarbamate (stressed mice). Doses mentioned are in mg/kg.
anxiety effect was observed to be higher than that produced by PDTC and diazepam alone in stressed mice (Figs. 1 and 2).

Effect of different treatments on plasma nitrite levels

Six-hour immobilization significantly increased plasma nitrite levels in mice as compared to vehicle-treated control mice (unstressed mice) (Fig. 3). Diazepam (2 mg/kg, i.p.) did not produce any significant change in plasma nitrite levels in unstressed mice and stressed mice (Fig. 3). SB-203580 (0.5, 1, or 2 mg/kg, i.p.) did not produce any change in basal plasma nitrite levels in unstressed mice as compared to vehicle-treated unstressed mice. In stressed mice, only high dose of SB-203580 (2 mg/kg, i.p.) significantly attenuated the immobilization stress–induced increase in plasma nitrite levels (Fig. 3). PDTC (100 mg/kg, i.p.) did not produce any change in basal plasma nitrite levels in unstressed mice as compared to

![Fig. 2.](image-url) Effect of different treatments on percentage of time spent by mice in light compartment of light/dark box. Values are expressed as mean ± S.E.M. (n = 6 in each group). Data were analyzed by one-way ANOVA followed by Tukey’s post hoc test, P < 0.0001. a = P < 0.05, significant difference from vehicle-treated control group (unstressed mice); b, c, d, e = significant difference from immobilization group (stressed mice); c = P < 0.05, significant difference from diazepam-treated stressed mice and SB-203580–treated stressed mice; e = significant difference from diazepam-treated stressed mice and PDTC-treated stressed mice. Veh: vehicle, IMMO: immobilization, DZP(U): diazepam (unstressed mice), DZP(S): diazepam (stressed mice), SB(U): SB-203580 (unstressed mice), SB(S): SB-203580 (stressed mice), PDTC(U): pyrrolidine dithiocarbmate (unstressed mice), PDTC(S): pyrrolidine dithiocarbmate (stressed mice). Doses are in mg/kg.

![Fig. 3.](image-url) Effect of different treatments on plasma nitrite levels (micromoles/liter). Values are expressed as mean ± S.E.M. (n = 6 in each group). Data were analyzed by one-way ANOVA followed by Tukey’s post hoc test, P < 0.0001. a = P < 0.05, significant difference from vehicle-treated control group (unstressed mice); b, c, d, e = significant difference from immobilization group (stressed mice); c = P < 0.05, significant difference from the immobilization group (stressed mice); e = P < 0.05, significant difference from the immobilization group (stressed mice). Veh: vehicle, IMMO: immobilization, DZP(U): diazepam (unstressed mice), DZP(S): diazepam (stressed mice), SB(U): SB-203580 (unstressed mice), SB(S): SB-203580 (stressed mice), PDTC(U): pyrrolidine dithiocarbmate (unstressed mice), PDTC(S): pyrrolidine dithiocarbmate (stressed mice). Doses are in mg/kg.
vehicle-treated unstrained mice. In stressed mice, PDTC (100 mg/kg, i.p.) significantly attenuated the immobilization-stress-induced increase in plasma nitrite levels (Fig. 3). SB-203580 (2 mg/kg, i.p.) pre-treatment did not produce any change in plasma nitrite levels in diazepam (2 mg/kg, i.p.)-treated unstrained mice. On the other hand, SB-203580 (2 mg/kg, i.p.) pretreatment significantly attenuated the immobilization-induced increase in plasma nitrite levels in diazepam (2 mg/kg, i.p.)-treated stressed mice. The noted decrease in plasma nitrite levels were higher than that produced by SB-203580 (2 mg/kg, i.p.) alone in stressed mice (Fig. 3). PDTC (100 mg/kg, i.p.) pre-treatment did not produce any change in plasma nitrite levels in diazepam (2 mg/kg, i.p.)-treated unstrained mice. On the other hand, PDTC (100 mg/kg, i.p.) pretreatment significantly attenuated the immobilization-induced increase in plasma nitrite levels in diazepam (2 mg/kg, i.p.)-treated stressed mice. The noted decrease in plasma nitrite levels were higher than that produced by PDTC (100 mg/kg, i.p.) alone in stressed mice (Fig. 3).

Effect of different treatments on locomotor activity of mice

The doses of different treatments producing significant anti-anxiety effects in the present study did not produce any significant effect on locomotor activity of mice, as compared to their respective controls. Therefore, the said results are not shown.

Discussion

Forced immobilization combines emotional stress (escape reaction) and physiological stress (muscle work). As painful stimuli are not directly involved in restraint stress, this form of stress is probably more akin to physiological stress (31). Belzung and Griebel (32) have reported that continuous exposure to different stressors result in enhancement of normal anxiety. We have also used physical immobilization for 6 h as a stressor for mice and found that stress-exposed mice were more anxious in their behavior, when tested on EPM and LDT, as compared to unstrained mice. This finding is in agreement with earlier reports (5–8, 25).

Acute immobilization stress, as used in the present study, is reported to increase expression of iNOS in the brain cortex and leads to production of the stable nitric oxide metabolites (nitrite and nitrate) in both plasma and brain (4, 33). The stressor used in the present study (6-h immobilization) has been found to increase TNF-α levels (4). A transgenic mouse model, overexpressing the gene for TNF-α has been shown to express excessive (pathological) anxiety in the LDT (34). In the present study, the LDT was utilized in addition to EPM as a test for measurement of anxiety in previously immobilized mice. The increase in TNF-α levels is implicated in NF-κB activation and iNOS expression in the brain cortex after immobilization stress (6 h), as used in the present study (4). Inhibition of iNOS by a nonselective inhibitor like l-NAME (5, 35) or by relatively selective inhibitors of nNOS, like 7-nitroindazole (36, 37), produced an anti-anxiety-like effect. Recently, anxiolytic-like activity of aminoguanidine, an inhibitor of the inducible isoform of NOS (7), was reported in mice from our own laboratory. Further, we observed a differential role played by neuronal and inducible isoforms of NOS in anxiety in mice under unstrained and stressed conditions (25).

Sources for nitric oxide overproduction caused by immobilization stress include elevated cytokines (38). Cytokines are reported to induce nitric oxide synthase enzyme expression through activation of p38MAPK (13, 39, 40). It is reported that 6-h immobilization stress, as used in the present study, is involved in the expression of iNOS in rat brain cortex via the release of TNF-α and has been hypothesized to be responsible for anxiety in stressed mice (4). Furthermore, activation of the transcription factor NF-κB, that is, the nuclear transport of NF-κB subunits, p50/p65, p50/p50, has been identified as an essential requirement for the expression of iNOS leading to an increase in NO production (16). Acute stress (6 h) has been demonstrated to stimulate the translocation of the NF-κB to the nucleus, and the administration of the NF-κB inhibitor PDTC at the onset of stress inhibits the stress-induced increase in iNOS expression (15). Earlier, in our laboratory, PDTC was found to produce antianxiety-like activity in previously immobilized mice (25). NF-κB is a central mediator for induction of genes for iNOS in response to physical stress (41, 42). PDTC has been found to reach higher and long-lasting intracellular concentrations and potently interferes with the mobilization of NF-κB in intact cells (43). PDTC (75 and 150 mg/kg, i.p.) inhibits immobilization stress–induced increase in iNOS expression (15). In the present study, PDTC (100 mg/kg, i.p.) per se produced an anti-anxiety effect in stressed mice, but not in stressed mice. Further, PDTC served to enhance the anti-anxiety effect in stressed mice. The per se effect of PDTC is in agreement with our earlier findings (25).

In this study, immobilization-induced stress induced a significant attenuation in the anxiolytic effect of diazepam in well-known behavioral models of anxiety, such as the EPM and LDT. Diazepam (2 mg/kg) produced a significant anxiolytic-like effect in unstrained mice, but could not exert significant anxiolysis in stressed mice. This effect is independent of its effect on locomotor activity,
as noticed in the present study and also supported by the literature (44). This is the only report showing the insignificant antianxiety effect of diazepam at a dose of 2 mg/kg in immobilization (6 h)-induced stressed mice. Reduction of GABAergic tone was found in stressed animals (45, 46, 47). The above reports are further supported by studies on diazepam viz. L-Arginine (100 mg/kg, i.p.), assumed to increase the synthesis of NO, has been reported to abolish the anxiolytic-like effect of diazepam (2 mg/kg, i.p.) (48).

p38MAPK inhibitors are efficacious in several disease models, including inflammation, arthritis and other joint diseases, septic shock, and myocardial injury (49, 50). In the present study, SB-203580 (2 mg/kg) per se produced a significant anti-anxiety effect and restored the diminished anxiolytic effect of diazepam significantly. There exists sufficient experimental evidence in different pathologies that NF-κB might be an effector of p38MAPK (51–53). As mentioned already above, 6-h immobilization stress has been well reported to activate NF-κB, which is an essential requirement for the expression of iNOS (15). Sources for nitric oxide overproduction caused by immobilization stress include elevated cytokines (38). Six-hour immobilization stress, as used in the present study, is responsible for elevation of TNF-α levels (4, 15, 34). Moreover, Cytokines are reported to induce nitric oxide synthase expression through activation of p38MAPK (13, 39, 40). Looking at these sequences of biochemical events and the present findings with SB-203580 and PDTC, it may be suggested that p38MAPK may also activate NF-κB in the present model of stress (6-h immobilization)-induced anxiety.

In the present study, diazepam failed to bring any change in nitrite levels in both unstressed and stressed mice. This inability of diazepam to modify the stress-induced increased nitriergic influence may be responsible for the compromised effect of diazepam in stressed mice. Furthermore, observations with SB-203580 and PDTC indicate that the compromised anxiolytic effect of diazepam in stressed conditions can be unmasked by inhibition of p38MAPK and NF-κB. The findings of the present study may further be strengthened by future studies with other drugs in the benzodiazepine category.

References
17. Weizman AM, Bidder FF, Gavish M. Food deprivation modulates gamma-butyric acid receptors and peripheral benzodiazepine binding sites in rats. Brain Res. 1990;535:96–100.


