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Short communication

Modulation of stress-induced neurobehavioral changes by nitric oxide in rats

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Abstract

The involvement of nitric oxide (NO) in stress-induced neurobehavioral changes in rats was evaluated using the elevated plus maze and open field tests. Restraint stress (1 h) reduced both the number of entries and time spent in open arms, with both expressed as percent of controls (no restraint stress), and these changes were reversed with diazepam (1 mg/kg) and the NO precursor, L-arginine (500 and 1000 mg/kg) pretreatment. The nitric oxide synthase inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME) (50 mg/kg), aggravated restraint stress effects in the elevated plus maze test, whereas the lower dose (10 mg/kg) of the drug attenuated the same. In the open field test, the restraint stress-induced (a) increased entry latency and (b) decreased ambulation and rearing were reversed by diazepam and L-arginine and L-NAME (10 mg/kg), whereas L-NAME (50 mg/kg) aggravated restraint stress effects. The neuronal nitric oxide synthase inhibitor, 7-nitroindazole (10 and 50 mg/kg), did not influence these restraint stress-induced behavioral changes to any significant extent. Biochemical data showed that L-NAME (10 and 50 mg/kg.) induced opposite effects on the total brain nitrate/nitrite content during restraint stress. The results indicate a possible involvement of NO in stress-induced neurobehavioral effects.

Keywords: Stress; Nitric oxide (NO); Elevated plus maze; Open-field

1. Introduction

Stress is conceived as any physical, psychological and/or environmental stimulus capable of altering physiological homeostasis, and the ability to cope with such stressful stimuli is a crucial determinant of health and disease (Selye, 1936; Bohus et al., 1990). The concept of stress has now evolved into one of a 'stress system' wherein complex interactions between central nervous system viz. the limbic system, the hypothalamo-pituitary-adrenal axis and several components of the visceral system (e.g. gastric mucosa) occur to a variety of stress inputs and such interactions between them plays a significant role in the outcome of the stress response (Chrouses and Gold, 1992; Ray et al., 1988, 1992a,b, 1993). Complex neural networks are also known to regulate the activity of the stress system and drugs modulating neurotransmitter/neuromodulators exert differential effects on the response of the various organs/systems (Ray et al., 1992a,b; Henke, 1987; Henke and Ray, 1992).

Nitric oxide (NO), a stable gaseous free radical, is now recognized as an important biomodulator. Several physiological functions have been attributed to NO and it has also been implicated in various pathological states. NO is also recognized as an intracellular messenger in central nervous system and its role as a neurotransmitter/neuromodulator has been proposed (Moncada et al., 1991; Gairthwaite et al., 1988; Zhang and Snyder, 1995). NO can be generated in the neurons and neuronal nitric oxide synthase and NMDA receptor activation can predispose to the occurrence of this (Gairthwaite et al., 1988; Zhang and Snyder, 1995). Some neurobehavioral effects of NO are documented and its role in analgesia, convulsion and memory has been proposed (Zhang and Snyder, 1995). NO has also been associated to anxiety, but the results of this experimental study are equivocal to say the least (Lino De Oliviera et al., 1997; Volke et al., 1998). However, the role of NO in stress and stress related behavioural changes is not clearly defined. The

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present study was therefore designed to evaluate the role of NO in some stress-induced neurobehavioral effects in rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (150–200 g) were used in the study. Rats were housed at a constant temperature of 20 + 2 °C under a 12-h light:12-h dark cycle. The animals ($n = 6–10$ per group) had free access to food and water throughout the experiments. Animal care was as per Indian National Science Academy (INSA) Guidelines for the Care and Use of Animals in Scientific Research, and the study had the approval of Institutional Animal Ethical Committee (IAEC).

2.2. Stress procedure

The animals were subjected to restraint stress for 1 h at room temperature by immobilizing them in adjustable Plexiglas restrainers (INCO, Ambala). Immediately after the restraint stress procedure, the rats were exposed to the behavioral tests.

2.3. Drugs

The following drugs were used: L-arginine hydrochloride, *N*-nitro L-arginine methyl ester (L-NAME), 7-nitroindazole (7-NI) and Diazepam (all from Sigma Aldrich, USA). L-Arginine hydrochloride and L-NAME were dissolved in distilled water. Diazepam and 7-nitroindazole were suspended in distilled water with a drop of Tween 80. All drugs were freshly prepared and administered intraperitoneally (i.p.) in a volume of 1 ml/kg. The pretreatment time for diazepam, L-NAME and 7-nitroindazole was 30 and 120 min for L-arginine.

2.4. Elevated plus maze test

The elevated plus consisted of two opposite arms 40 × 40 crossed with two similar closed arms with walls of 40-cm height. The arms were connected so that the maze had a plus sign look. The entire maze was elevated 50 cm above ground level and placed in a quiet, dimly lit room (Pellow et al., 1985; Bhattacharya and Satyan, 1997). Naive or pretreated rats were placed individually in the center of the maze facing the closed arms. The following parameters were measured: number of open-arm entries, and time spent on open-arm entries and closed-arm entries. Subsequently, the percentages of open-arm entries and time spent on open arms were calculated from open-arm entries and time spent on open arms divided by the total number of entries in both open and closed arms and time spent on open arm exploration divided by total time spent in both open and closed arms, respectively.

2.5. Open field test

The open field apparatus consisted of a square arena 96 × 96 cm with 60-cm-high walls. The walls painted were white and the floor green. The floor was divided into 16 squares by parallel and intersecting white lines (Bhattacharya and Satyan., 1997; Carli et al., 1989). Rats were placed singly in one corner of the open field and (a) latency, (b) ambulation and (c) rearing were observed during a 5-min exposure period for both naïve and pretreated animals.

2.6. Brain nitrates and nitrites (NOx) assay

Brain NO*x* contents were determined as described by Tracey et al. (1995). Brain samples were homogenized in 5-ml distilled water and centrifuged at 10 000 × *g* for 15 min at 4 °C. Fifty microlitres of supernatant was mixed with 20 μl of 0.11 mM FAD and 20 mU of nitrate reductase. Samples were allowed to incubate for 1 h at room temperature in the dark. Then 5 μl of 1 M $ZnSO_4$ was added to the samples in order to precipitate the proteins. Samples were centrifuged at 6000 × *g*, for 5 min at 4 °C and the supernatants were removed. One hundred microlitres of Griess reagent (1:1 mixture of 1% sulphanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)-ethylenediamine) was added to 50 μl of supernatant and the mixture was incubated for 10 min at room temperature. Absorbance was measured at 540 nm in a microassay plate by microscan MS 5605A (Electronics Corporation of India) and converted to NO*x* content using a nitrate standard curve. Brain supernatant protein was estimated by Lowry's method (Lowry et al., 1951). The data were expressed as nmol NO*x*/mg protein.

2.7. Statistics

The data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test for post hoc comparisons. A *P* value of at least 0.05 was considered as the level of significance in all statistical tests.

3. Results

3.1. Effects of stress and NO modulators on elevated plus maze test in rats

Analysis of elevated plus maze data revealed that percent (%) numbers of both entries and time spent in open arms were significantly different across all groups (viz. restraint stress and no restraint stress), $F(16,102) = 10.21$, $P < 0.001$ and $F(16,102) = 8.71$, $P < 0.001$, respectively (one-way ANOVA). Restraint stress (1 h) induced a significant reduction in the open-arm entries (%) ($P < 0.01$, Dunnett's test), whereas the data for the percent time spent in the open arms were not statistically significant (Table 1). In the intergroup comparisons, pretreatment with diazepam (1 mg/kg)

Table 1
Effect of restraint stress (RS) and of Nitric Oxide (NO) modulators on Elevated Plus Maze test parameters in rats

Treatment (mg/kg i.p.)	Elevated plus maze parameters (mean ± S.E.)		
	n	Open-arm entries (%)	Time spent (%)
Vehicle (no RS)	10	23.2 ± 2.3	10.3 ± 1.2
Vehicle + RS	8	5.0 ± 3.2[a]	1.2 ± 1.1
Diazepam (1)	6	58.1 ± 3.1[b]	20.6 ± 2.4
Diazepam (1) + RS	8	41.6 ± 6.0[c]	12.7 ± 1.7[d]
L-Arginine (500)	6	43.5 ± 4.6	12.1 ± 1.4
L-Arginine (500) + RS	9	28.2 ± 3.0[c]	15.9 ± 1.3[c]
L-Arginine (1000)	6	48.3 ± 4.7[a]	13.0 ± 1.5
L-Arginine (1000) + RS	10	41.3 ± 3.9[c]	21.4 ± 3.3[c]
L-NAME (10)	7	40.2 ± 10.7	11.7 ± 5.7
L-NAME (10) + RS	10	38.0 ± 2.5[c]	26.2 ± 5.1[c]
L-NAME (50)	6	11.0 ± 8.3	3.6 ± 2.9
L-NAME (50) + RS	10	5.5 ± 3.0	1.0 ± 0.5
7-Nitroindazole (10)	6	28.5 ± 9.8	10.6 ± 5.2
7-Nitroindazole (10) + RS	6	11.0 ± 6.9	2.5 ± 1.8
7-Nitroindazole (50)	7	15.4 ± 8.2	3.3 ± 1.6
7-Nitroindazole (50) + RS	7	9.7 ± 4.6	2.0 ± 1.3

[a] $p < 0.05$—compared to Vehicle (no RS) group.
[b] $p < 0.01$—compared to Vehicle (no RS) group.
[c] $p < 0.01$—compared to Vehicle + RS group.
[d] $p < 0.05$—compared to Vehicle + RS group.

reversed the restraint stress-induced changes in elevated plus maze activity and both open-arm entries and percentage time spent in open arms were markedly greater than those in the vehicle + restraint stress group ($P < 0.01$ and $P < 0.05$, respectively). L-Arginine (500 and 1000 mg/kg) prior to restraint stress significantly increased the percentage number of entries and also increased the time spent in the open arms ($P < 0.01$), the effect on both parameters being dose-dependent. A lower dose of L-arginine (100 mg/kg) did not produce a similar response in open-arm activity, and though there was a 27% increase in the time spent in the open arms when compared to the vehicle + restraint stress group, these differences were not statistically significant ($P > 0.05$, data not shown). On the other hand, pretreatment with the nitric oxide synthase inhibitor, L-NAME (10 and 50 mg/kg), modified the restraint stress effects on the elevated plus maze parameters in a dose-related manner. The lower (a) dose (10 mg/kg) increased both no. of entries and time spent in the open arms significantly ($P < 0.01$), whereas the (b) higher dose (50 mg/kg) markedly reduced open-arm entries, when compared to the vehicle + restraint stress group. Another nitric oxide synthase inhibitor, 7-nitroindazole (10 and 50 mg/kg), when given prior to restraint stress exposure, showed similar trends as seen with L-NAME (10 mg/kg), and though there was an appreciable increase in elevated plus maze activity, the data were not statistically significant ($P > 0.05$). In non-stressed rats (no restraint stress), diazepam enhanced elevated plus maze activity markedly ($P < 0.01$) and similar effects were seen with L-arginine (500 and 1000 mg/kg) ($P < 0.01$). However, none of the nitric oxide synthase inhibitors was able to influence elevated plus maze parameters to any significant extent. These results are summarized in Table 1.

3.2. Effects of stress and NO modulators on the open-field behavior in rats

Analysis of open-field test data showed that (a) latency of entry, (b) ambulations and (c) rearings were significantly different across all (restraint stress and no restraint stress) groups ($F(16,102) = 13.18$, $P < 0.001$ for latency, $F(16,102) = 7.03$, $P < 0.001$ for ambulation, and $F(16,102) = 14.89$, $P < 0.001$ for rearing, respectively; one-way ANOVA). Restraint stress (1 h) induced a marked increase in the latency (s) of entry into the open field, whereas ambulation and rearing were appreciably reduced, the data for the latter two parameters being statistically significant ($P < 0.01$, Dunnett's test) (Table 2). Pretreatment with diazepam (1 mg/kg) attenuated the restraint stress effects on the open field behavior, i.e. latency of entry was decreased, and both ambulation and rearing activity were increased. L-Arginine (500 and 1000 mg/kg), given prior to restraint stress, also showed diazepam-like effects, i.e. the latency of entry was reduced and there was a general increase in both ambulatory and rearing activity ($P < 0.01$) in each case. L-NAME (10 and 50 mg/kg) induced dose-related effects on restraint stress-induced open field behavior. Whereas the lower dose of L-NAME (10 mg/kg) reduced latency of entry and increased ambulation and rearing ($P < 0.01$), the higher dose (50 mg/kg) further aggravated the restraint stress-induced behavior in the open-field, primarily manifested as marked increase in entry latency ($P < 0.01$), when compared to that of the vehicle + restraint

Table 2
Effect of restraint stress (RS) and of Nitric Oxide (NO) modulators on Open-Field test parameters in rats

Treatment (mg/kg i.p.)	Open field parameters (mean ± S.E.)			
	n	Latency (s)	Ambulation	Rearing
Vehicle (no RS)	10	4.5 ± 0.8	40.6 ± 5.1	21.2 ± 2.3
Vehicle + RS	10	10.5 ± 1.2	7.7 ± 1.4[a]	7.7 ± 1.2[a]
Diazepam (1)	6	0.6 ± 0.1	64.5 ± 2.9	28.3 ± 1.6
Diazepam (1) + RS	6	0.8 ± 0.1	82.0 ± 14.0[b]	48.6 ± 5.0[b]
L-Arginine (500)	6	1.5 ± 0.2	45.6 ± 1.4	20.6 ± 1.7
L-Arginine (500) + RS	9	2.3 ± 0.9	52.5 ± 10.8[b]	18.8 ± 1.5[c]
L-Arginine (1000)	6	1.1 ± 0.1	52.6 ± 3.1	20.0 ± 2.1
L-Arginine (1000) + RS	10	2.2 ± 1.7	48.3 ± 5.3[b]	33.1 ± 2.6[b]
L-NAME (10)	6	1.3 ± 0.2	48.0 ± 7.6	22.6 ± 3.7
L-NAME (10) + RS	9	2.0 ± 0.4	56.4 ± 11.6[b]	26.1 ± 3.0[a]
L-NAME (50)	6	12.3 ± 0.9[a]	11.5 ± 0.7	7.6 ± 2.2[b]
L-NAME (50) + RS	9	62.5 ± 14.6[b]	17.2 ± 6.9	12.1 ± 4.0
7-NI (10)	6	1.6 ± 0.2	46.8 ± 9.2	18.5 ± 4.4
7-NI (10) + RS	6	2.0 ± 0.2	31.1 ± 5.5	10.6 ± 2.0
7-NI (50)	7	3.1 ± 0.4	36.5 ± 4.6	10.7 ± 1.5
7-NI (50) + RS	6	3.7 ± 0.5	24.0 ± 2.9	8.0 ± 1.1

[a] $p < 0.01$—compared to Vehicle (no RS) group.
[b] $p < 0.01$—compared to Vehicle + RS group.
[c] $p < 0.05$—compared to Vehicle + RS group.

stress group. Further, pretreatment with 7-NI (10 and 50 mg/kg) induced a reduction in open field entry latency and an increase in ambulatory activity these data were not statistically significant ($P>0.05$) when compared to those for the vehicle + restraint stress group. In addition, the higher dose of L-NAME (50 mg/kg), which induced behavioral suppression in both elevated plus maze and open field tests, did not induce any appreciable sedation or catalepsy, when compared to the vehicle-treated group. In non-stressed rats, none of the drugs used was able to influence the open field test parameters to any significant extent, except for L-NAME (50 mg/kg), with which open field latency was increased and rearing activity decreased ($P<0.01$), when compared to vehicle (no restraint stress) group.

3.3. Effect of restraint stress and L-name on brain NO activity

Biochemical assay for total nitrates and nitrites (NO*x*) in the brain supernatant showed that L-NAME (10 and 50 mg/kg) had different (and even opposite) effects on brain NO*x* in restraint stress-exposed rats. Whereas in the vehicle + restraint stress group the values for NO*x* were 0.473 ± 0.034 nmol/mg protein, the values for the L-NAME (10) + restraint stress and L-NAME (50) + restraint stress groups were 0.613 ± 0.050 nmol/mg protein (a 28% increase compared to restraint stress) and 0.273 ± 0.024 nmol/mg protein (a 43% inhibition compared to restraint stress). The brain NO*x* data were significantly different across all groups—$F(3,15)=20.28$, $P<0.001$ (one-way ANOVA)—and post-hoc comparisons by Dunnett's test showed that the data for both L-NAME groups were significantly different from the data for vehicle + restraint stress RS group ($P<0.05$).

4. Discussion

Environmental factors like stress can influence the neurobehavioral profile of the organism and precipitate an anxiety-like syndrome, and behavioral factors such as emotionality are useful predictors of stress susceptibility (Henke et al., 1991; Handley and Blane, 1993). Experimental pharmacological studies have shown that anti-anxiety agents attenuate a variety of autonomic, visceral and immunological responses to stress and the present study shows that classical anti-anxiety agents such as diazepam (1 mg/kg) markedly reversed the stress-induced behavioural changes in the elevated plus maze and the open-field test in rats. Both elevated plus maze and open field tests have been used very effectively to test the neurobehavioral profile of animals under the influence of anxiogenic/anxiolytic agents (Carli et al., 1989; Bhattacharya and Satyan, 1997). In the elevated plus maze test, increases in both (a) percentage number of entries and (b) percentage time spent in the open arms are indices of anxiolytic activity, and our results with diazepam (1 mg/kg) are consistent with an anti-stress effect of the agent. Similarly, in the open field test, diazepam (1 mg/kg) reduced entry latency and increased ambulation and rearing behavior.

A neuromodulatory role of nitric oxide (NO) has been speculated upon and experimental studies have shown that NO may play a crucial role in central nervous system (CNS)-related disorders (Zhang and Snyder, 1995). Further, stress (emotional and environmental) is known to be a key factor in the genesis of neurological and psychiatric illness (Chrouses and Gold, 1992). However, the role of NO in stress and stress-induced changes is not well documented. Stress-induced changes are regulated by complex neurohumoral mechanisms in the CNS and, in view of the close similarity between anti-anxiety and anti-stress effects and the increasing evidence for the role of NO as a chemical messenger in the CNS, the effects of NO modulators were assessed in these two experimental models of anxiety in order to assess the role of NO in stress-induced behavioral changes.

The NO precursor, L-arginine, consistently reversed the restraint stress-induced suppression of (a) percentage open-arm entries and percentage time spent in the open arms in the elevated plus maze and (b) the decreased latency of entry and increase in rearing/ambulation in the open field test. These effects were very similar to those seen with diazepam and thus are highly suggestive of an anti-stress/adaptogenic profile for NO. These findings receive support from the observation that diazepam and L-arginine effects had a similar trend/nature in both elevated plus maze and open field tests in non-stressed rats. The observation that inhibition of NO synthesis by L-NAME (50 mg/kg, i.p.) produced opposite effects on restraint stress-induced changes in elevated plus maze and open field activity is consistent with this hypothesis. These changes were very similar and even greater than those seen with restraint stress alone, indicating that inhibition of NO synthesis could be associated with an anxiogenic or stress-like effect. Further, since L-NAME (50 mg/kg) did not have any significant effect on general behavioral parameters such as sedation and catalepsy, it is likely that the drug influenced or interacted with the stress system to induce these behavioral effects.

Some of our present results, however, are not totally in agreement with some results of earlier studies which show anti-anxiety effects for L-NAME in the elevated plus maze test in normal rats (Faria et al., 1997). The differences in the results could have been due to the additional influence of stress (restraint stress) under which the drug effects were observed in the present study. However, the fact that the restraint stress and NO modulators produced consistent effects in both elevated plus maze and open field open field tests support our hypothesis on the anti-stress/adaptogenic role of NO. Interestingly, low doses of L-NAME (10 mg/kg) attenuated the restraint stress-induced neurobehavioral effects in both elevated plus maze and open field tests. Further, the neuronal nitric oxide synthase inhibitor, 7-NI, did not significantly influence ($P>0.05$) most of the behavioral parameters in the elevated plus maze and open field

open field tests, both in either normal or restraint stress situations, indicating that the NO generated from pathways other than via neuronal nitric oxide synthase could also be involved in the NO-stress interactions. The paradoxical effect of L-NAME (10 mg/kg) could also possibly be due to partial inhibition of overall NO activity in the brain, resulting in levels of NO adequate to produce anti-stress effects, and qualitatively similar to those seen with the anti-stress agent, diazepam. Biochemical data also showed that, in L-NAME (10 mg/kg)-treated animals, the NO*x* levels in the brain supernatants was higher than in either vehicle treated or L-NAME (50 mg/kg)-treated animals, after restraint stress (see results), suggestive of higher levels of NO activity in the CNS. Taken together, the results of the present study indicate that NO may act as an endogenous anti-stress agent/adaptogen in the CNS and that NO-ergic mechanisms could play a crucial modulatory role in stress-induced neurobehavioural effects.

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