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# Pharmacological and biochemical studies on the possible role of nitric oxide in stress adaptation in rats

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**Abstract**

The involvement of nitric oxide (NO) in stress adaptation was evaluated in rats using the elevated plus maze test. Repeated restraint stress RS( × 5) for 5 days resulted in an increase in the percentage number of entries and percentage time spent when compared to a single restraint stress RS( × 1) exposure. In the repeated RS treatment groups, the nitric oxide donor, L-arginine (500 and 1000 mg/kg, i.p.) slightly increased the elevated plus maze test parameters when compared to the corresponding vehicle-treated group. The nitric oxide synthase (NOS) inhibitors, *N*-nitro-L-arginine methyl ester (L-NAME, 10 and 50 mg/kg, i.p.) and 7-nitroindazole (10 and 50 mg/kg, i.p.) produced differential responses in both the parameters with L-NAME exhibiting greater reduction in open arm entries and open arm time, whereas 7-nitroindazole produced only small differences in both the elevated plus maze parameters. Biochemical data showed that repeated restraint stress resulted in higher levels of brain nitrates and nitrites (NO*x*) as compared to that of single restraint stress exposure. Further, in L-arginine (1000 mg/kg, i.p.)-treated rats, brain NO*x* was lowest in the single restraint stress group, followed by repeated restraint stress and (no restraint stress) controls. The results are suggestive of the role of nitric oxide in stress adaptation and this may be due to the effects of restraint stress on brain NOS activity.


*Keywords:* Stress; NO (Nitric oxide); Elevated plus maze; Adaptation

## 1. Introduction

Stress is an aversive stimulus which disturbs physiological homeostasis (Selye, 1936) and the effect of stress is reflected on a variety of biological systems (Gairthwaite et al., 1988). Exposure to a stressor is known to evoke responses such as reduced locomotor activity, marked anorexia, decreased growth rate and hypersecretion of corticosterone in animals and humans (Chrouses and Gold, 1992). Behavioral studies have shown that repeated exposure to a stressor results in a diminished stress response as compared to the stress response induced by the initial exposure (Burchfield et al., 1988; Kennet et al., 1985) .This desensitization to the stressor during repeated exposure is referred to as stress adaptation, a mechanism believed to protect an organism against the impact of repeated stress exposures.

Several studies on the neurochemical basis underlying stress adaptation have shown changes in enzymes which regulate the synthesis of neurotransmitters (e.g. cathecholamines) as well as the density of neurotransmitter receptors (Anisman et al., 1984; Irwin et al., 1986). For example, some studies have demonstrated that 2.5 h/day repeated restraint stress for 7 days resulted in a decrease in [$^3$H]dihydroalprenolol binding sites which positively correlated with the appearance of stress adaptation (Stone and Platt, 1982; Stone et al., 1985). Nitric oxide, a unique diatomic molecule, has been attributed a number of important biological functions. In the central nervous system, it acts as an intracellular messenger as well as a neurotransmitter (Moncada et al., 1991; Gairthwaite et al., 1988; Zhang and Snyder, 1995).

In view of the above, the present study was designed to evaluate the role of nitric oxide (NO) in the repeated stress-

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induced/adaptive changes by studying the stress–NO interactions in the elevated plus maze test. Further the total nitrates and nitrites (NO*x*), which are stable nitric oxide metabolites, were measured for corroborative purposes in the brain. Earlier studies from our laboratory have indicated the involvement of nitric oxide in acute stress-induced neurobehavioral and biochemical changes and an anti-stress role for nitric oxide has been suggested (Masood et al., 2003). However, the role of NO in the mechanism underlying repeated stress-induced behavioral changes is less known.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats (150–200 g) were used in the study. Regular rat chow and tap water were allowed ad-libitum. 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. All procedures were done in accordance with 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 (IEAC).

### 2.2. Stress procedure

The animals were subjected to restraint stress (RS) 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 test. Both single restraint RS( × 1) and repeated restraint RS( × 5) procedures were used for the experiment.

### 2.3. Drugs

The following drugs were used :-L-arginine hydrochloride, *N*-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (all from Sigma Aldrich, USA.). L-arginine hydrochloride and L-NAME were dissolved in distilled water. 7-Nitroindazole was 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 L-NAME and 7-nitroindazole was 30 min and 120 min for L-arginine respectively.

### 2.4. Elevated plus maze

The elevated plus maze consisted of two open arms 40 × 40 cm 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, time spent on open-arms and closed-arm entries. Subsequently, the percentage of open-arm entries and time spent on open arms was 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. Brain nitrates and nitrites( NOx) assay

Brain nitrates and nitrites (NO*x*) content 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 0.86 mM β-NADPH, 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 microplate reader (MS5605A, ECIL) 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.

### 2.6. Statistics

The data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test, for post hoc multiple comparisons. Trend analysis by regression was also done at appropriate places by ANOVA. A value of at least 0.05 was considered as the level of significance in all statistical tests. The analysis was carried out by using SPSS.

## 3. Results

### 3.1. Effects of restraint stress and nitric oxide modulators in the elevated plus maze test

Analysis of the elevated plus maze data revealed that the percentage number of open-arm entries and time spent in the open arm were statistically significant across all groups. ($F(8,53) = 5.103$, $P < 0.05$ and $F(8,53) = 5.46$, $P < 0.05$, respectively, one-way ANOVA). Acute (single) restraint stress RS( × 1) exposure induced a significant reduction in the

Table 1
Effects of single RS( × 1) and repeated RS( × 5) restraint stress on elevated plus maze activity and brain NO*x* in rats

| Treatment (mg/kg, i.p.) | Elevated plus maze parameters (Mean ± S.E.) | | | Brain NO*x* (Mean ± S.E.) |
|---|---|---|---|---|
| | *n* | %Open-arm entries | %Open-arm time | nM/mg protein |
| Control | 10 | 26.3 ± 3.6 | 10.3 ± 1.2 | 1.1 ± 0.09 |
| RS( × 1) | 8 | 6.4 ± 3.3* | 1.3 ± 1.0** | 0.47 ± 0.01* |
| RS( × 5) | 6 | 30.8 ± 11.9* | 12.0 ± 5.2** | 1.0 ± 0.06** |
| L-Arg-500 RS( × 5) | 7 | 33.3 ± 4.4 | 15.8 ± 1.6 | 0.9 ± 0.07 |
| L-Arg-1000 RS( × 5) | 6 | 46.6 ± 4.7 | 12.1 ± 2.0 | 1.2 ± 0.11 |
| L-NAME-10 RS( × 5) | 6 | 14.1 ± 1.1 | 7.3 ± 3.1 | 0.96 ± 0.07 |
| L-NAME-50 RS( × 5) | 6 | 32.0 ± 11.0 | 4.4 ± 2.0 | 1.1 ± 0.12 |
| 7-NI-10 RS( × 5) | 7 | 38.1 ± 2.0 | 15.8 ± 1.6 | 1.3 ± 0.18 |
| 7-NI-50 RS( × 5) | 6 | 38.8 ± 1.9 | 12.2 ± 2.0 | 1.6 ± 0.28 |

Control, no RS; L-Arg, L-arginine; L-NAME, *N*-nitro-L-arginine methyl ester; 7-NI, 7-nitroindazole.

*$p < 0.01$ (Compared to control group).

**$p < 0.01$ (Compared to RS( × 1) group).

open arm entries and time spent, ($P < 0.01$ and $P < 0.05$, respectively, (Dunnett's test) (Table 1). Repeated restraint stress RS( × 5) exposure however had lesser inhibitory effects as compared to that of single restraint stress RS( × 1) viz. there was a relative increase in the percentage number of open arm entries and percentage time spent in the open arms, and the difference was statistically significant in both the elevated plus maze parameters ($P < 0.01$ and $P < 0.05$, respectively, Dunnett's test.)

L-arginine (500 and 1000 mg/kg) attenuated the repeated restraint stress (RS) induced changes viz. there was an increase in the percentage number of open arm entries and percentage time spent in the open arms, but these differences were not statistically significant when compared to corresponding vehicle-treated group.

Analysis of the elevated plus maze data with NO-modulators per se revealed that the percentage number of open arm entries and percentage time spent in the open arms were statistically significant across all groups. ($F(6,39) = 3.817$ and $F(6,39) = 3.891$, $P < 0.05$, respectively, one-way ANOVA. L-arginine produced significant effects in both the elevated plus maze test parameters ($P < 0.05$), but the rest of the NO modulators were not able to bring about a marked change.

The nitric oxide synthase inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME, 10 and 50 mg/kg, i.p.) induced decreases in the percentage number of open arm entries, but though there was a 39.1% and 63.3% decrease in the open arm time at these dose levels, respectively, these differences did not achieve statistical significance when compared to corresponding vehicle-treated group. The nitric oxide synthase inhibitor, 7-nitroindazole(10 and 50 mg/kg, i.p.), on the other hand, produced marginal enhancements in both the elevated plus maze test parameters when compared to repeated vehicle-treated group exposed to repeated restraint stress (RS), but the differences were not statistically significant ($P > 0.05$).

Trend analysis by regression was done with three dose levels (0, 500 and 1000 mg/kg) for L-arginine on the open arm entries and with three dose levels (0, 10 and 50 mg/kg) for L-NAME on the open arm time but significance ($P < 0.05$) was achieved only in the L-arginine treatment group with a *b*-coefficient of 0.476.

### 3.2. Effect of stress and nitric oxide modulators on total brain nitrates and nitrites (NOx)

Analysis of the biochemical data showed that the total nitrates and nitrites (NO*x*) in the brain supernatants were statistically significant across all groups ($F(8,53) = 5.571$, $P < 0.05$ one-way ANOVA ). The total NO*x* in the brain supernatant showed that single restraint stress RS( × 1) exposure induced a significant decrease ($P < 0.01$, Dunnett's test) when compared to vehicle-treated rats. On the other hand, repeated restraint stress RS( × 5), brain NO*x* was higher when compared to the corresponding single restraint stress RS( × 1) data, the differences being statistically significant ($P < 0.05$, Dunnett's test ). In the repeated restraint stress RS( × 5) situation, L-arginine (1000 mg/kg, i.p.) only marginally increased total brain NO*x* when compared to the corresponding vehicle group. However, the lower dose L-arginine (500 mg/kg, i.p.) was not able to attenuate the repeated restraint stress RS( × 5) induced changes in the brain NO*x* values. Both L-NAME and 7-nitroindazole (10 and 50 mg/kg, i.p.) produced only slight increases in this brain NO*x* and the differences were not statistically significant when compared to vehicle controls.

A comparison between behavioral and biochemical data is shown in Fig. 1. Both open arm entries and brain NO*x* were suppressed after single restraint RS( × 1) exposure, whereas both parameters were back to near control (no RS) levels after repeated restraint RS( × 5).

To further validate these experimental data, rats were administered L-arginine (1000 mg/kg, i.p.) and were divided

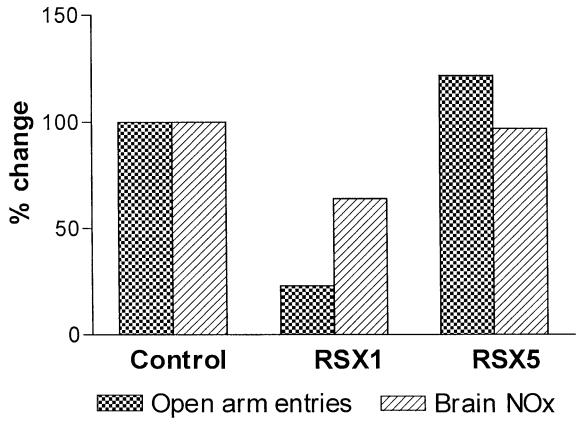


Fig. 1. Relationship between open arm entries (OAE) and brain NO*x* in single RS( × 1) and repeated restraint stress RS( × 5) exposed rats. Results are expressed as percentage change. Control = no RS; RS = Restraint stress. Control values of open arm entries and brain NO*x* are taken as 100%.

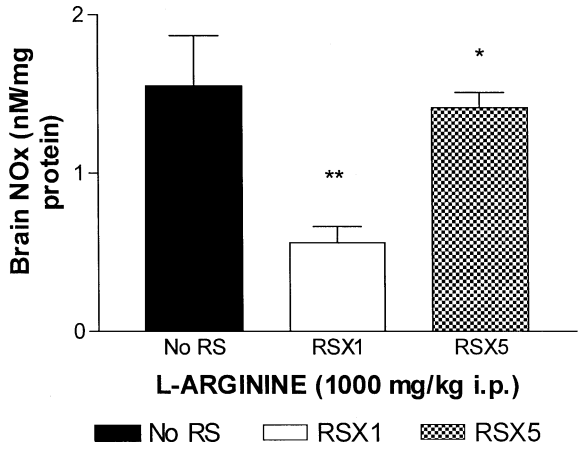


Fig 2. Effect of single RS( × 1) and repeated RS( × 5) on the brain NO*x* in L-arginine-treated rats. Results are expressed as nM/mg protein. *$P<0.05$, **$P<0.01$ as compared to the No RS and RSx1 group, respectively. RS=restraint stress.

into three groups, which were separately exposed to (a) no restraint stress (no RS), (b) single restraint stress RS( × 1) and (c) repeated restraint stress RS( × 5) sessions and subsequently total brain nitrates and nitrites (NO*x*) were measured. In rats exposed to single restraint stress RS( × 1) session, brain NO*x* was the lowest, followed by the group which was exposed to repeated restraint stress RS( × 5) sessions, and the no RS group had the highest brain NO*x*. Analysis of the data showed that these differences were statistically significant across all the groups ($F(2,17)=6.087$, $P<0.05$, one-way ANOVA ) and inter-group comparisons showed that brain NO*x* in the single restraint stress RS group was significantly different for both no restraint stress (no RS) and repeated restraint stress RS( × 5) groups ($P<0.05$, Dunnett's test in both cases). These results are summarized in Fig 2.

## 4. Discussion

The present study demonstrates that repeated restraint stress RS( × 5) exposure of rats results in behavioral adaptation as measured by the elevated plus maze test. The significant finding of this study was that single and repeated restraint stress treatments induced differential changes in (a) behavior and (b) brain nitrates and nitrites (NO*x*). When rats were exposed to a novel environment such as the elevated plus maze, they usually show a clear preference for the closed arms, but do show some open arm activity. Consistent with this finding, we found that control unstressed animals showed increased number of entries and time spent in the open arms. However, when rats were previously subjected to repeated restraint, they moved more frequently and spent more time in the open arms. According to the present study, it seems that repeated restraint stress exposure induces behavioral adaptation which probably favors the release of suppressed behavior. Thus, when exposed to an elevated plus maze, repeatedly restraint RS( × 5) rats were less susceptible to behavioral suppression than those exposed to a single restraint stress session.

Stress is known to be a key factor in the genesis of neuropsychiatric disorders and adaptation to stress helps in the coping process (Cancela et al., 1991). Nitric oxide has been speculated to be an important neuromodulator in experimental conditions including stress (Masood et al., 2003), but the role of NO in stress adaptation has not been fully understood.

A recent study from our laboratory has confirmed that L-arginine, a NO precursor, consistently reversed restraint stress (RS) induced suppression of the percentage number of entries and time spent in open arms in the elevated plus maze and this effect was mimicked by diazepam, a finding that is highly suggestive of an anti-stress role for NO. This finding received further support from the fact that NOS inhibitor, L-NAME, further aggravated the restraint stress (RS)-induced behavioral suppression (Masood et al., 2003).

In the repeated restraint stress RS( × 5) treatment groups, L-arginine also attenuated (albeit marginally) the restraint stress RS( × 5)-induced changes in behavior and brain NO*x*. This supports our previous contention suggesting an anti-stress profile for NO. In the present study, this attenuating effects of three different dose levels of L-arginine on repeated restraint stress-induced changes on the elevated plus maze parameters, which was confirmed by trend analysis, further supported this concept.

The NO synthase inhibitor, L-NAME, on the other hand, further aggravated the repeated restraint stress RS( × 5)-induced behavioral changes and brain NO*x*. This could be due to the relative increase in NO synthase activity due to repeated restraint stress RS( × 5) probably as an adaptive/protective mechanism, thereby partially allowing L-NAME to block the active sites of the activated NO synthase enzyme, and hence not profoundly affecting behavior and total brain nitrates and nitrites (NO*x*).

The suppression of NO synthase activity by single restraint stress RS( × 1) and removal of this suppression by repeated restraint stress RS( × 5) exposures is further validated by the significant decrease in the brain NO*x* after L-arginine (1000 mg/kg) in the single restraint stress RS( × 1) group, when compared to L-arginine treated, no RS group. This indicates that perhaps nitric oxide synthase activity is suppressed by single RS which prevents the conversion of L-arginine to nitric oxide in the brain. However, in repeated RS group, L-arginine-induced NO formation was higher indicating that the suppression of NO synthase was gradually being removed, resulting in a relatively increased NO synthase activity and increased brain NO*x*. This may be due to the fact that repeated RS-induced adaptation resulted in gradual removal of the NO synthase suppression that may have been induced by a single RS exposure. An earlier study (Kishimoto et al., 1996) has reported that nNOS is upregulated by acute stress (6 h). Our results with repeated RS are generally along similar lines and suggest that both the intensity and duration

of the stressor could play an important role in stress. Interestingly, the NO synthase inhibitors L-NAME and 7-nitroindazole induced differential nature of effects on both EPM parameters and total brain NO*x*. This may be due to the fact that other isoforms of the NO synthase enzymes (other than nNOS) could be involved in stress. This concept has also been forwarded in an earlier study from our laboratory (Masood et al., 2003).

Taken together, the present pharmacological and biochemical data indicates that NO may play an important role in stress adaptation and this, along with the previous report implicating NO in acute stress, supports the involvement of nitric oxide in stress reactions.

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