Involvement of nitric oxide (NO) in the regulation of stress susceptibility and adaptation in rats

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The present study evaluated the regulatory role of nitric oxide (NO) in stress susceptibility and adaptation in rats. Acute restraint stress (RS ×1) reduced the number of entries and time spent in the open arms in the elevated plus maze (EPM) test and raised plasma corticosterone levels. RS (×1)-induced neurobehavioral suppression and raised corticosterone levels were attenuated by pretreatment with the NO precursor, L-arginine (500 and 1000 mg/kg) and unaffected or further aggravated by NO synthase inhibitor, L-NNAME or 7-nitroindazole (10 and 50 mg/kg). Biochemical assay of plasma and brain homogenates showed that these RS – induced behavioral and neuroendocrinal changes were associated with lowered levels of plasma and brain total nitrates/nitrites (NOx). L-Ar ginine attenuated the RS-induced suppression of NOx levels in plasma and brain, whereas, the NO synthase inhibitors tended to produce reverse effects. In the experiments involving repeated stress i.e. RS (×5), exposure resulted in attenuation/reversal of (a) neurobehavioral suppression in the EPM test and (b) lowered brain NOx, that was seen after RS (×1). The RS (×5)-induced changes in EPM parameters and brain NOx were further potentiated after L-arginine pretreatment, whereas, the NO synthase inhibitors were less effective. Rats were screened as high and low emotional in the open-field test, and high emotional rats showed greater (a) behavioral suppression in the EPM, (b) corticosterone responses (c) brain NOx suppression, and (d) cold-restraint stress (CRS) induced gastric mucosal lesions as compared to their low emotional counterparts. L-Arginine pretreatment was more effective in modulating the above RS induced stress responses/markers in the high emotional group of rats. Our data suggest that NO plays a differential role during exposure to acute and repeated stress situations, and that the relationship between stress and emotionality status may be under the regulatory influence of NO.

Keywords: Adaptation, Emotionality, Nitric oxide, Stress

Stress is known to disturb the physiological homeostasis of the organism and the ability to cope with such stressful stimuli are crucial determinants of health and disease. Since the introduction of the concept of “stress” to biomedical research by Hans Selye1,2 stress research has evolved considerably over the last few decades, and cellular and molecular concepts are being forwarded to explain stress responses. Complex neurochemical pathways have been demonstrated to regulate stress responses and interactions between the host factors that are instrumental in deciding the nature and the extent of the impact of such aversive inputs on the biological system. In fact, the concept of a “stress system” is being strongly advocated in which a holistic approach involving interactions between CNS, neuroendocrine, immune and visceral systems are being explored3-13. For example, factors like emotionality and prior stress exposure are also known to influence the stress response, and adaptation to stress is known to occur after repeated exposures to such aversive stimuli7,8,14.

Nitric oxide (NO), a ubiquitous free radical moiety, which was first discovered in the vascular endothelium, is now known to be distributed in many tissue/organ systems and its role as a neuromodulator/neurotransmitter in the CNS has been documented. The role of NO in several pathophysiological states have been suggested and NO modulators are effectively used as therapeutic agents in medicine15-23. However, the role of NO in stress and stress responses is still not clearly defined. In view of the above, in the present study we have critically evaluated the possible role for NO in acute and repeated stress exposure situations using several multi-system markers of stress responses in rats. Further, the possible role of NO in the relationship between emotionality and stress susceptibility was also assessed.

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Materials and Methods

Male Wistar rats (175-200g), 6-8 per group, were used for the study and the protocol was approved by the Institutional Animal Ethical Committee. The animals had free access to food and water and were housed in standard laboratory conditions as per animal care guidelines of the INSA. Restraint stress (RS) in plexiglas restrainers (INCO, Ambala) for 1 hr at room temperature was used as the experimental stressor and depending upon the study design, single RS (×1) and repeated sessions of RS (×5) were employed. In the experiments involving repeated RS, rats were exposed to RS sessions daily for 1h for 5 consecutive days, followed by assessment of the various stress responses after the last RS session. Cold restraint stress (for 3h at 4º C, CRS) was used for gastric ulcer studies.

Neurobehavioral studies

Elevated plus maze (EPM) test

This apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × height 40 cm), facing each other, with a central open square area at the intersection of these arms and elevated 50 cm above ground level – thus giving it a “plus” (+) shaped appearance. The animals were placed in the central square and observed for 5 min, and the number of open/closed arm entries as well as the time spent in the open/closed arms were recorded during this 5 min exposure. RS was employed immediately before EPM exposure. In the repeated RS sessions, the animals were exposed to 5 sessions of RS daily for 1h, and the effects were seen as above on the EPM test after the last RS session.

Open-field (OF) test

In this test, emotionality rating of the animals were tested by exposing them to the open-field (OF) apparatus, which is a square wooden area with the floor divided into 16 squares and having high walls, with a dim light (electric bulb, 10W) from the top. The animals were placed in one corner square, and the following parameters were noted - (a) the latency of entry into the main arena of the OF; (b) the number of squares crossed; (c) number of rearings; and (d) number of defecation pellets, during a 5 min exposure to OF. If the rat defecated before 5 rearings took place, the rat was labeled as high emotional and if not, then as low emotional.

Biochemical assay

Immediately after termination of RS procedure and subsequent behavioral tests, blood was collected by cardiac puncture for the estimation of plasma corticosterone and total nitrates/nitrites (NOx) assays. Then, rats were quickly sacrificed and the brains were removed aseptically. The cerebella were discarded and the rest of the brain was washed in cold water and saline. The brain tissue was homogenized in 0.1 M phosphate buffer (pH 7.4) as per standard procedure, and the brain homogenates thus prepared were stored at −20°C till estimations were performed.

Plasma corticosterone—Plasma corticosterone was assayed by the method of Mattingly26 with some modifications. Briefly, blood samples were centrifuged at 1000×g for 10 min at 4ºC and plasma was removed. To 200 μl of plasma, 1.5 ml of dichloromethane was added and centrifuged at 5000×g for 5 min. After discarding the top aqueous layer, 500 μl of fluorescent reagent (absolute ethanol + conc. sulphuric acid) was added and shaken. After 20 min the fluorescence of the bottom layer was measured at 470 nm (excitation) and 530 nm (emission) using a spectrofluorimeter (Perkin-Elmer) and fluorescence was translated to the corticosterone values (μg/dl) by using the standard curve.

NO assay (NOx)—For NO assay estimation, NOx (stable NO metabolites) was estimated in plasma and brain homogenate. Samples were assayed spectrophotometrically (ECIL, India) using the method of Tracey et al.27. Briefly, the plasma and brain homogenates were treated with nitrate reductase + NADPH + FAD to convert all nitrates to nitrites. After incubation for 1 h at 37ºC in the dark, zinc sulphate was added to precipitate the proteins. After centrifuging (6000×g), the supernatants were transferred to 96 well assay plates and Griess reagent (1 : 1 mixture of 1% sulfanilamide in 3% orthophosphoric acid and 0.1% naphthyl ethylene diamine) was added for color development. The plates were then read at 540 nm by UV spectrophotometer (ECIL), and NOx was calculated by using a standard curve constructed for this purpose. Brain supernatants were assayed for protein content28 and data were expressed as nm NOx/mg of protein.

Gastric ulcers

Stress gastric ulcers were induced by exposing 18h food deprived rats to restraint stress in plexiglas restrainers for 3h at 4ºC (CRS). Immediately after the RS procedure rats were sacrificed with an overdose of ether. The stomachs were dissected out, cut open along the greater curvature, washed in cold water, and
examined under a microscope (×10) for number and severity (cumulative length in mm) of gastric lesions11–13.

Drugs and chemicals

The drugs and chemicals used were - L-arginine hydrochloride, L-NAME, 7-nitroindazole, corticosterone, dichloromethane, sulphuric acid, FAD, NADPH, nitrate reductase, sulphanilamide, naphthyl ethylene diamine, orthophosphoric acid (the latter three combined as Griess reagent). All drugs were dissolved in distilled water, except for 7-nitroindazole (7 – NI) which was dissolved in a drop of Tween-20 and volume made up by distilled water. They were injected intraperitoneally (ip) in a volume of 2 ml/kg following appropriate pretreatment schedules, i.e. 120 min for L-arginine and 30 min for L-NAME or 7-NI and this was on the basis of our earlier studies in this area29.

Statistical analysis

Data have been expressed as mean ± SE and the experimental groups were compared with respective control groups for statistical significance. The statistical analysis was done using Mann-Whitney U test. P value of at least 0.05 was considered as the level of significance in all statistical tests.

Results

Restraint stress (RS) consistently suppressed the number of open arm entries in the EPM test, and this was associated with reductions in the time spent in the open arms, during the 5 min exposure to the test. These values in the RS exposed group were appreciably lower as compared to the control (no RS) group and these differences were statistically significant. Pretreatment of rats with NO precursor, L-arginine (500 and 1000 mg/kg) dose dependently attenuated the RS-induced behavioral suppression in the EPM test. In fact, the results with the higher dose were more consistent and of higher magnitude than that of the control group. On the other hand, administration of the NO synthase inhibitor, L-NAME (10 and 50 mg/kg) prior to RS induced behavioral inhibition similar to that observed in the vehicle + RS group. The time spent in the open arm in response to L-NAME (50 mg/kg)+RS was further suppressed than that of vehicle+RS group. The pattern of activity in the EPM test after 7-NI (a nNOS inhibitor) was similar to L-NAME i.e. both doses of 7-NI induced neurobehavioral suppression, similar to that seen with vehicle+RS. RS also induced a significant rise in plasma corticosterone levels as compared to the control (no RS) group and these differences were statistically significant. L-arginine dose dependently attenuated the corticosterone response to RS and with L-arginine (1000 mg/kg) the levels were rather lower than the vehicle+RS group. On the other hand, both NO synthase inhibitors, L-NAME and 7-NI, when administered prior to RS, induced effects similar to those seen with vehicle+RS i.e. corticosterone levels were elevated. These results have been summarized in Table 1.

Biochemical assay of the brain homogenates and plasma for Nox showed that brain and plasma NOx levels were significantly lower in RS exposed rats as compared to controls. Further, pretreatment with the NO precursor, L-arginine, reversed brain Nox changes seen after RS and these values were significantly different from that of the vehicle + RS group. On the other hand, brain and plasma NOx levels after 7-NI pretreatments were similar to those seen in the vehicle + RS group of rats. However, administration of L-NAME (50 mg/kg) prior to RS further suppressed the NOx levels in plasma as compared to that of vehicle+RS group. These results have been summarized in Table 2.

In studies involving repeated stress RS(×5), there was a remarkable reversal of behavioral suppression seen in EPM as compared to the single restraint (RS ×1) group. The number of open arm entries as well as the time spent on open arms of the EPM of RS (×5)
group were significantly greater than those of the RS (×1) group. Pretreatment with L-arginine (1000 mg/kg) further enhanced the reversal, whereas L-NAME or 7-NI (both at 50 mg/kg) were ineffective; rather they tended to attenuate the reversal seen after repeated RS (×5). Brain NOx levels were also significantly higher in the RS (×5) group when compared to the data of the RS (×1) group. As seen with the behavioral data, L-arginine attenuated the biochemical responses to RS i.e. raised the brain NOx levels as compared to the respective vehicle treated group, whereas, both L-NAME and 7-NI did not appreciably influence this biochemical marker for NO. These results have been summarized in Table 3.

In the next set of experiments, rats were screened for their emotionality status by exposing them to the open field (OF) test. On the basis of rearing/defecation ratio, the animals were screened as high emotional if the ratio was < 5 and low emotional if the ratio was = or > 5. Differences in the stress responses were observed on neurobehavioral and biochemical parameters, in both groups. As seen earlier, RS(×1) induced suppression in (a) EPM open arm entries and (b) brain NOx levels, and these suppressions were higher in the high emotional rats as compared to their low emotional counterparts. In high emotional rats, L-arginine (1000 mg/kg) treatment, prior to RS, significantly attenuated the neurobehavioral suppression seen in the vehicle + RS group. This was also accompanied by appreciable increase/reversal of brain NOx levels. However, these effects were not so evident in low emotional rats. L-NAME pretreatment did not affect the EPM activity as well as brain NOx levels after RS in high emotional rats, whereas these effects were further suppressed in low emotional rats. These results have been summarized in Table 4.

High emotional rats had more severe gastric lesions as compared to the low emotional group, when both groups were exposed to cold + restraint stress (CRS) after 18 h of food (but not water) deprivation. Pretreatment with L-arginine (1000 mg/kg) consistently attenuated gastric ulcer severity in both groups of rats, the changes being more marked in the high emotional group. L-NAME (50 mg/kg), on the other hand, did not affect the gastric ulcerations in the present study.

### Table 3—Effects of repeated restraint stress (RS ×5) and NO modulators on neurobehavioral and endocortical stress markers and brain total nitrites/nitrites (NOx) in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Elevated PlusCorticosterone (ug/dl)</th>
<th>Brain Nox (n mole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arg – L-Arginine; L-NAME – L-nitroarginine methyl ester; 7-NI – 7-nitroindazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + RS (×1)</td>
<td>7.8±2.0</td>
<td>0.86±0.20</td>
</tr>
<tr>
<td>L-Arg (1000) + RS</td>
<td>16.8±3.3</td>
<td>25.2±3.1</td>
</tr>
<tr>
<td>L-NAME (50) + RS</td>
<td>10.6±2.5</td>
<td>38.0±6.6</td>
</tr>
<tr>
<td>7-NI (50) + RS</td>
<td>12.4±1.8</td>
<td>30.5±5.5</td>
</tr>
<tr>
<td>Controls (no RS)</td>
<td>23.6±3.1</td>
<td>18.4±2.2</td>
</tr>
</tbody>
</table>

*P < 0.05; P < 0.02 (compared to the respective vehicle + RS group)

### Table 4—Effects of stress (RS) and NO modulators on EPM activity and brain NOx levels in high emotional and low emotional rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EPM activity</th>
<th>Brain Nox</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arg – L-Arginine; L-NAME – L-nitroarginine methyl ester; 7-NI – 7-nitroindazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + RS</td>
<td>5.5±1.1</td>
<td>0.72±0.20</td>
</tr>
<tr>
<td>L-Arg (1000) + RS</td>
<td>22.8±4.2</td>
<td>2.00±0.42</td>
</tr>
<tr>
<td>L-NAME (50) + RS</td>
<td>11.2±6.0</td>
<td>1.02±0.30</td>
</tr>
<tr>
<td>Controls (no RS)</td>
<td>20.8±3.6</td>
<td>1.85±0.46</td>
</tr>
</tbody>
</table>

*P < 0.05; P < 0.02 (compared to the respective vehicle + RS group)

### Table 2—Effects of acute restraint stress (RS) and NO modulators on plasma and brain nitrates and nitrites (NOx) in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma NOx (n mol/ml)</th>
<th>Brain NOx (n mol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + RS</td>
<td>1.00±0.08</td>
<td>0.86±0.20</td>
</tr>
<tr>
<td>L-Arg (1000) + RS</td>
<td>1.60±0.20</td>
<td>1.72±0.09</td>
</tr>
<tr>
<td>L-NAME (50) + RS</td>
<td>0.58±0.25</td>
<td>0.74±0.10</td>
</tr>
<tr>
<td>7-NI (50) + RS</td>
<td>0.92±0.25</td>
<td>1.25±0.30</td>
</tr>
<tr>
<td>Controls (no RS)</td>
<td>1.82±0.15</td>
<td>2.10±0.30</td>
</tr>
</tbody>
</table>

L-Arg – L-Arginine; L-NAME – L-nitroarginine methyl ester; 7-NI – 7-nitroindazole

*P < 0.05 (compared to the vehicle + RS group)
other hand, consistently aggravated stress gastric lesions in high but not in low emotional group of rats. These results have been summarized in Table 5.

Discussion

The present results clearly indicate that NO plays an important regulatory role in the susceptibility and adaptation to stress. In the neurobehavioral studies, RS suppressed EPM activity, which is a reliable and sensitive measure for testing effects of anxiogenic/anxiolytic treatments. Decreased open arm entries/time is suggestive of anxiety like states and increased open arm entries/time is suggestive of anxiolytic activity. The fact that RS induced changes in EPM activity were reversed by L-arginine and unaffected/aggravated by L-NAME suggests that lowered NO activity was responsible for the stress-induced anxiogenic effect. The hypothalamo-pituitary-adrenal axis mediated elevations in plasma corticosterone are consistent and sensitive markers of stress responsiveness. In the present study, RS markedly elevated plasma corticosterone levels and these were attenuated by L-arginine, but not by NO synthase inhibitor pretreatment. Behavioral and neuroendocrinal responses in response to RS and their modulation by the NOergic agents are strongly suggestive of the role of NO during stress reactions. Earlier studies have, however, indicated the possible relationship between HPA axis activity and NO, but the association of this interaction with stress was not clearly defined. Our findings are further corroborated by the observation that during such RS, both plasma and brain NOx activity were lower than controls, indicating that RS effects on behavior and corticosterone might be associated with lower NO levels, probably via inhibition of NO synthases. NO is a highly labile and reactive molecule and gets quickly converted to the more stable nitrates and nitrites (NOx), which are good markers for NO activity/levels. Biochemical data showed that NOx levels were lower in both brain and plasma in the RS exposed group. These changes were attenuated/reversed by L-arginine pretreatment. The fact that the NO synthase inhibitors, L-NAME or 7-NI were unable to influence NOx levels appreciably, may be due to the fact that maximum suppression of NOx would already have occurred after RS, and hence the NO synthase inhibitor effects were not apparent.

Interestingly, the results of the experiments involving repeated RS (RS×5) were quite different from those seen after single RS (×1) exposures. RS (×5) induced changes which were far lesser in intensity as compared to single RS treatment. The reduced anxiogenic response i.e. greater number of open arm entries and more time spent in EPM after RS (×5) as compared to RS (×1) suggested that such exposure could induce adaptation/tolerance to the stressful stimuli, which was in turn reflected on the neurobehavioral profile of the animals in this group. The neurochemical contribution of NO to this phenomenon is evident as brain NOx levels were elevated in RS (×5) group of rats as compared to the single RS (×1) group. Henke has also reported that repeated exposure to stressor induces adaptative reactions which are manifested as reduction in the magnitude of physiological and behavioral changes. In the present study, RS(×5) exposed rats exhibited reversals in (a) behavioral inhibition, (b) corticosterone responses and (c) brain NOx suppression, when compared to the single RS (×1) situation. These results, when considered alongwith the acute RS (×1) data, strongly suggested that both, acute stress reactions and repeated stress induced adaptative responses were under the regulatory influence of NO. Chronic stress induce enhanced expression of NOS in the rat cortex has been reported and our findings appear to be in agreement with this hypothesis.

Table 5—Effect of NO modulators on stress-induced gastric ulcer formation in high emotional and low emotional rats

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Gastric ulcer severity (mm)</th>
<th>High emotional</th>
<th>Low emotional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + CRS</td>
<td>3.2±0.61</td>
<td>1.8±0.4</td>
<td></td>
</tr>
<tr>
<td>L-Arg (1000) + CRS</td>
<td>1.6±0.4 a</td>
<td>1.0±0.4 a</td>
<td></td>
</tr>
<tr>
<td>L-NAME (50) + CRS</td>
<td>5.0±1.0 a</td>
<td>2.2±0.8</td>
<td></td>
</tr>
<tr>
<td>Controls (no CRS)</td>
<td>0.4±0.2 b</td>
<td>0.2±0.2 b</td>
<td></td>
</tr>
</tbody>
</table>

L-Arg – L-Arginine ;L-NAME – L-nitroarginine methyl ester 
P< 0.05; b P< 0.02 (compared to the vehicle + CRS group)

Emotionality has been suggested to be an important predictor of stress susceptibility. In the open-field test, rats were screened for their emotionality status, and the fact that the high emotional rats had lower brain NOx as compared to their low emotional counterparts, indicates emotionality and stress susceptibility are under the regulatory control of NO as well. Thus, neurobehavioral and biochemical data indicate that NO may be involved in both stress...
susceptibility and adaptation. Stress gastric ulcers are widely recognized as emotional responses to stressors. In the present study, CRS induced gastric ulcers of more severity in the high emotional group as compared to the low emotional group of rats. Such stress ulcers were attenuated by L-arginine and aggravated by L-NAME, providing pharmacological evidence for the role of NO in this stress response.

Taken together, the present pharmacological and biochemical data indicate that acute and repeated stress-induced changes in behavioral and physiological parameters as well as the interactions between stress and emotionality, are under the regulatory influence of NO. The results also suggest that NO may act as an endogenous adaptogen and be a good target molecule for drug development in stress and anxiety.

Acknowledgement

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References

