Differential effects of nitric oxide synthase inhibitors on anxiety in unstressed and stressed mice

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Effects of selective nitric oxide synthase (NOS) inhibitors, 7-nitroindazole (7-NI), a selective inhibitor of neuronal nitric oxide synthase (nNOS) and aminoguanidine (AG), a selective inhibitor of inducible nitric oxide synthase (iNOS) on anxiety in unstressed and stressed mice were investigated using elevated plus maze (EPM) test and light-dark test (LDT). 7-NI (20 and 40 mg/kg, ip) produced anti-anxiety effect in unstressed mice but not in stressed mice. AG (50 and 100 mg/kg, ip) produced anxiolytic effect in stressed mice and failed to produce the similar effect in unstressed mice. Nitrite levels were increased in stressed mice, but not in unstressed mice, exposed to EPM and LDT for 5 min. Increased nitrite levels in stressed mice were attenuated by AG, but not by 7-NI. The effects of AG were enhanced by pyrrolidine-dithio-carbamate (PDTC), an inhibitor of NF-κB induction, in stressed mice. The results suggest the possible role of inducible nitric oxide synthase in stress-induced anxiogenesis as compared to unstressed mice, where neuronal form of NOS may play predominant role.

Keywords: Anxiety, Immobilization, Nitric oxide synthase

Anxiety disorders are common mental disorders that share extreme or pathological anxiety as the primary disturbance in mood or emotional tone1. Diagnostic and Statistical Manual IV (DSM IV) describes various forms of anxiety disorders, including phobias, generalized anxiety, post-traumatic stress, panic and obsessive-compulsive disorders2. Common denomination of all anxiety disorders is a state of increased fear and exaggerated version of the acute stress response3,4. Biochemical alterations are observed in anatomical centres, which are responsible for processing of emotions and are connected to “stress” axis (HPA axis), which is strongly activated in response to threat or fear5,7.

Restrain is one of the best explored models of stress in rodents as this model combines both emotional (escape reaction) and physiological (muscle work) stress8. Further, it also create a situation, in which normal anxiety (experienced by animal in animal models) is expressed in excess and this stress—potentiated behaviour serve to be better measure of anxiety because it is enhanced anxiety state that is physiologically close human pathological feature, clinically more relevant and covers broad spectrum of anxiety disorders including phobia, post-traumatic stress disorder and panic9. Chronic stress may be a main factor in development of anxiety10. Exposure to various stressors result in anxiogenic behavior in tests of anxiety in animals11. Exploration of open arms in elevated plus maze is decreased by stressors like forced swim12, surgical stress13, social defeat14 and immobilization15. Exposure of rats to elevated plus maze (EPM) has been observed to activate brain regions related to anxiety and activate nitric oxide synthase (NOS)16. Both the above conditions of (a) exposure to animal model (normal anxiety) and (b) restraint (excess anxiety) present two different types of anxiety. Different forms of anxiety have been shown to respond differentially to pharmacological interventions17.
A number of neurochemicals have been shown to modulate anxiety-related behaviors. Among them, nitric oxide (NO) is well positioned to modulate the hypothalamic–pituitary–adrenal (HPA) axis. NO, an intercellular messenger in the brain, plays an important role in various physiological and pathological processes. NO is generated from L-arginine, by various NADPH-dependent enzymes called nitric oxide synthases (NOS). NO and stress physiology share many common pathways and molecular mechanisms, and NO plays role in many stress-related diseases. Stress strongly involves activation of NOS and NO-cascade leading to behavioral changes. However, a differential modulation is shown by NO in response to stressful stimuli e.g. intracerebroventricular or peripheral injection of the non-selective NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), attenuates stress-induced adreno-corticotrophic hormone (ACTH) release. In contrast, pretreatment with L-NAME increases plasma ACTH and corticosterone following peripheral lipopolysaccharide (LPS) or intravenous injections of cytokines. In stress conditions including physical restraint for 60 min or 120 min, enhanced anxious behavior is prevented by L-NAME in rodents. However, involvement of NO in anxiety is the most controversial as both anxiogenic and anxiolytic roles have been reported for nitric oxide. Almost all the investigations on involvement of NO in anxiety have targeted on either non-selective inhibition of NO or of neuronal isoform of NO (nNOS). Contrarily to the neuronal and endothelial NO synthases, iNOS is mainly regulated at the transcriptional level. iNOS is also expressed in brain cortex of rats exposed to acute and repeated stress. Inducible nitric oxide synthase (iNOS) is a high-output isoform of NOS which has been implicated in cellular toxicity in many cell systems including brain. Experimental evidences indicate that stress-induced iNOS expression in brain may mediate, at least in part, the anatomical and clinical features of neurotoxic damage found in animals and humans after exposure to uncontrollable stress. Aminoguanidine (AG) has been reported to be a selective inhibitor of iNOS. Recently, AG has been reported to produce antianxiety effect in stressed mice, accompanied by a decrease in plasma nitrite levels. An essential factor required for transcription of iNOS is NF-κB. Many experimental as well as clinical studies have documented an increased activity of NF-κB in pathological conditions of the CNS. Pyrrolidine-dithio-carbamate (PDTC)—an inhibitor of NF-κB potently interfere with the mobilization of NF-κB in intact cells. However, relative effects of relatively selective inhibitors of neuronal and inducible nitric oxide synthase on anxiety in unstressed and stressed rodents have not been explored. In the past years, enormous research efforts have been focused on developing mouse models of psychiatric disorders. Based on existing literature on effects of NOS inhibitors in anxiety and different effects of NO in stressed conditions, the present study has been designed to explore (a) whether nNOS inhibition will also produce anxiolysis under stress-enhanced anxiety conditions and (b) possible involvement of inducible isoform of NOS in anxiety behavior of mice in normal (animal model) and stressed (restraint) conditions. PDTC was used with the purpose to further explore whether blockade of induction of iNOS produce any difference in mice behavior on EPM and LDT (light-dark test). PDTC was also combined with inhibitors of iNOS and nNOS to observe their effect on unstressed and stressed mice behavior on EPM and LDT.

Materials and Methods

Animals—Swiss albino mice (22-30 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 food and tap water ad libitum and were exposed to 12 hr light and 12 hr dark cycle. The animals were acclimatized to the laboratory condition before experiments. 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. Experimental protocol was approved by Institutional Animal Ethics Committee.

Drugs—Aminoguanidine, 7-nitroindazole and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Elevated plus maze (EPM)—The plus maze apparatus consisted of two open arms (16 × 5 cm) and two closed arms (16 × 5 cm) enclosed by 12 cm high walls with an open roof, arranged so that the two open arms are opposite to each other. The maze was elevated to a height of 25 cm. Each mouse was placed individually at the centre of elevated plus maze with its head facing towards an open arm. During the 5 min
test, the number of entries into open arm, closed arm and time spent in each arm of the maze was recorded. The apparatus was thoroughly cleaned using 5% ethanol before placing each mouse in the cage.

**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. One compartment was painted black from inside and covered with a roof. The other compartment was open (no roof) and painted white. An animal was placed into the center of the light compartment and was observed for 5 min. The observations were made on time spent in open (white/light) compartment.

**Biochemical estimation of plasma nitrite**—Nitrite is the stable end product of nitric oxide (NO) in living systems. Accumulation of nitrite was measured by spectrophotometric assay based on Griess reaction. Blood was withdrawn from tail vein of mice and plasma was extracted using cooling centrifuge at 2500 rpm for 10 min. Plasma was mixed with equal volume of Griess reagent (1% sulphanilamide + 0.1% naphthylenediamine dihydrochloride + 2.5% phosphoric acid) and incubated at room temperature for 10 min to yield a chromophore. Absorbance was read at 543 nm spectrophotometrically (Beckman DU 640-B, Nyon, Switzerland). The nitrite concentration was calculated from standard curve using sodium nitrite as standard and expressed as micromolar of nitrite.

**Experimental design**—Mice (96) were divided into 16 groups of 6 mice each. Unstressed mice were exposed to EPM and LDT for normal duration (5 min), sufficient to assess the anxiety levels in rodents. Stress was produced in mice by immobilizing them for 6 h (0800-1400 hrs) by taping all its four limbs and trunk on a wooden board. Immobilized mice were called as stressed mice. Mice, not subjected to immobilization were called as unstressed mice and mentioned accordingly hereafter. However, for clarity of results and discussion, mice were clearly indicated as “vehicle- treated” or “drug-treated” unstressed or stressed mice throughout the manuscript. Behavioral testing was performed carefully in stepwise manner i.e. mice in each group were subjected to elevated plus maze followed by light and dark test. All the drugs were administered intraperitoneally (ip) 30 min before the behavioral testing in unstressed mice and immediately before immobilization in stressed mice. When combinations of drugs were employed, pre-treatments were administered 15 min earlier than other drugs. Behavioral testing was started 10 min after setting the animals free from immobilization and there was 5 min gap between two tests. Doses and routes of administration of drugs used in the present study have been adopted from studies utilizing similar studies for anxiety assessment i.e. aminoguanidine; 7-NI and PDTC. For nitrite estimations, blood samples were collected before subjecting the mice to behavioral testing. The dosage schedule assures that the treatment(s) inhibit any change(s) occurring immediately during immobilization, thereby producing the net change in behavior or biochemical parameter of mice under investigation.

**Statistical analysis**—All statistical analysis were done using one-way analysis of variance (ANOVA) in the Graph Pad Instat (GPIs) package, version 3.05.

**Results**

Physical immobilization (6 h) used in the present study served to enhance anxiety in mice, when tested on EPM and LDT as compared to vehicle treated unstressed mice (Tables 1 and 2). Immobilization also resulted in increased nitrite levels in mice (Table 3). In elevated plus maze model, unstressed mice showed preference towards closed arms as assessed by recording the time spent and number of entries in open and closed arms. Stressed mice (immobilized for 6 hr) showed significantly enhanced anxiety as evident by further decrease in time spent by mice in open arms of plus maze as compared to unstressed mice (P < 0.05). 7-NI (20 and 40 mg/kg, ip) produced anxiolytic effect in unstressed mice in elevated plus maze (increase in entries and time spent in open arms) as compared to vehicle treated unstressed mice (P < 0.001). However, 7-NI (20 mg/kg, ip and 40 mg/kg, ip) did not produced statistically significant anxiolysis in stressed mice in elevated plus maze. AG (50 mg/kg, ip and 100 mg/kg, ip) failed to increase the number of entries and time spent by unstressed mice in open arms of plus maze as compared to vehicle treated unstressed mice. An increase in time spent by mice in open arms of elevated plus maze was observed in AG (50 and 100 mg/kg, ip) treated stressed mice as compared to stressed mice (P < 0.001) (Table 1). PDTC per se produced anti-anxiety effect in stressed mice but not in unstressed mice. Administration of combination of 7-NI and PDTC (100 mg/kg, ip) in unstressed mice...
produced anti-anxiety effect as compared to unstressed mice \((P < 0.001)\). Administration of combination of AG (100 mg/kg, ip) and PDTC produced greater anti-anxiety effect than stressed group \((P < 0.001)\), AG (50 mg/kg, ip) treated stressed mice \((P < 0.001)\) and AG (100 mg/kg, ip) treated stressed mice \(P < 0.01\) (Table 1).

In light and dark test, unstressed mice did show preference towards dark compartment as assessed by recording the time spent in open and closed compartments. Stressed mice (immobilized for 6 hr) did show significantly enhanced anxiety as evident by further decrease in time spent by mice in open compartment of light/dark box as compared to unstressed mice \((P < 0.01)\). 7-NI (20 mg/kg, ip) \((P < 0.01)\) and 7-NI (40 mg/kg, ip) \((P < 0.001)\) produced significant anxiolytic effect in unstressed mice in light/dark test (increase in time spent in open compartment) as compared to vehicle treated unstressed mice. However, 7-NI (20 and 40 mg/kg, ip)
Table 3—Effect of different treatments on plasma nitrite levels as expressed in μM/L
[Values are expressed as mean ± SE of six animals in each group]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg, ip)</th>
<th>Plasma nitrite levels (μM/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh (U)</td>
<td>10 ml</td>
<td>10.3 ± 1.5</td>
</tr>
<tr>
<td>IMMO (S)</td>
<td>6 h</td>
<td>22.2 ± 2.2</td>
</tr>
<tr>
<td>5 min exposure to EPM</td>
<td></td>
<td>12.3 ± 1.6</td>
</tr>
<tr>
<td>5 min exposure to Light/dark Test</td>
<td></td>
<td>11.3 ± 2.4</td>
</tr>
<tr>
<td>7-NI (U)</td>
<td>20</td>
<td>12.9 ± 2.2</td>
</tr>
<tr>
<td>7-NI (S)</td>
<td>20</td>
<td>21.2 ± 2.4</td>
</tr>
<tr>
<td>7-NI (U)</td>
<td>40</td>
<td>13.9 ± 2.8</td>
</tr>
<tr>
<td>7-NI (S)</td>
<td>40</td>
<td>19.2 ± 1.4</td>
</tr>
<tr>
<td>AG (U)</td>
<td>50</td>
<td>11.0 ± 2.2</td>
</tr>
<tr>
<td>AG (S)</td>
<td>50</td>
<td>14.1 ± 1.2</td>
</tr>
<tr>
<td>AG (U)</td>
<td>100</td>
<td>14.6 ± 2.1</td>
</tr>
<tr>
<td>AG (S)</td>
<td>100</td>
<td>11.6 ± 1.1</td>
</tr>
<tr>
<td>PDTC (S)</td>
<td>100</td>
<td>9.8 ± 1.4</td>
</tr>
<tr>
<td>PDTC + 7-NI (U)</td>
<td>100 + 40</td>
<td>11.7 ± 2.6</td>
</tr>
<tr>
<td>PDTC + 7-NI (S)</td>
<td>100 + 40</td>
<td>11.2 ± 1.7</td>
</tr>
<tr>
<td>PDTC + AG (U)</td>
<td>100 + 100</td>
<td>9.8 ± 1.4</td>
</tr>
<tr>
<td>PDTC + AG (S)</td>
<td>100 + 100</td>
<td>9.0 ± 0.8</td>
</tr>
</tbody>
</table>

P values: \( a < 0.01 \): significant as compared to unstressed; \( b < 0.05 \); \( c < 0.01 \): significant as compared to stressed; \( d < 0.05 \); \( e < 0.001 \): significant as compared to stressed; \( f < 0.001 \): significant as compared to unstressed. Administration of combination of 7-NI and PDTC (100 mg/kg, ip) in stressed mice significantly decreased the nitrite levels as compared to vehicle treated stressed mice \((P < 0.05)\). On the other hand, administration of combination of AG (100 mg/kg, ip) and PDTC in stressed mice further decreased nitrite levels as compared to vehicle-treated stressed mice \((P < 0.001)\) (Table 3).

Discussion

Forced immobilization is one of the best explored models of stress in rats. This model combines emotional stress (escape reaction) and physiological stress (muscle work), resulting in both restricted mobility and aggression. As painful stimuli are not directly involved in restraint stress, this form of stress is probably more akin to physiological stress. Further, enhanced anxiety state is the more relevant and physiologically closer to behavior of anxious humans. Physical immobilization (6 h) as stressor for mice was also used and it was found that stress-exposed mice were more anxious in their behavior, when tested on EPM and LDT as compared to unstressed mice. Belzung and Griebel have discussed that continuous exposure to different stressors results in enhancement of normal anxiety. Maladaptive behavioral responses to these stressors results in longer lasting anxiety. Stressor used in the present study (6 h immobilization) has been found to increase TNF-alpha levels. A transgenic mouse...
model overexpressing gene for TNF-α has been shown to express excessive (pathological) anxiety in light and dark test. In the present study, light/dark test was also used in addition to EPM as a test for measurement of anxiety in previously immobilized mice. Both EPM and LDT are behaviorally and pharmacologically validated animal models of anxiety used for years to test anxiety. Moreover, EPM and light and dark test offer a similar environment of light and dark transition, against which the animals have been tested for behavioral responses. Therefore, this combination of tests in series may serve to enhance the robustness of the procedure. However, reports exploring the effects of relatively longer duration stress (24-72 h). For example, 72 h sleep-deprived mice, placed on small platform showed an anxiogenic behavior in elevated plus maze, whereas, opposite results were observed by another study, pointing to reduced anxiety in mice induced by similar 24 h small platform-stress. Inhibition of NOS has been earlier reported to result in anxiolysis in rodents, subjected for behavioral testing for 5 min on the models like EPM and LDT. These observation stand true in the results of present study too, as 7-NI (nNOS inhibitor) at doses of 20 and 40 mg/kg produced anti-anxiety effects in mice subjected to EPM and LDT procedures for 5 min. iNOS is quite selectively inhibited by AG. AG (50 and 100 mg/kg) used in the present study decreased immobilization–induced anxiogenic behavior in both EPM and LDT. These observed effects of AG support the involvement of iNOS in immobilization stress-induced damage in rodents. iNOS is responsible for formation of large amounts of nitric oxide. In the present study too, plasma nitrite levels were increased in vehicle treated stressed mice. These results are supported by recent report on antianxiety effect of AG, a selective iNOS inhibitor, where AG-induced anxiolysis was accompanied by a parallel decrease in plasma nitrite levels in mice. These results suggest the involvement of iNOS in antianxiety effect of AG. Expression of nNOS is also found to increase after restraint stress. But, 7-NI, a selective nNOS inhibitor, failed to produce anxiolysis in stressed mice. A suitable explanation to this finding may be that nNOS is more sensitive to autoinhibitory effects of NO on enzyme activity. 7-NI has also failed to block the stress-induced hippocampal NOS activation. This may be reason why 7-NI produce antianxiety effect in unstressed mice and failed to do so in stressed mice. It is observed that overproduction of NO following stress more likely involve iNOS and not nNOS. Further, increased nNOS expression following restraint stress may be more effectively targeted by 7-NI under conditions of severe stress, and may not do so in acute stress, used in the present study as suggested by these findings. NF-κB is a central mediator of the induction of genes for iNOS in response to a physical stress. PDTC has been found to reach higher and longer-lasting intracellular concentrations and potently interfere with the mobilization of NF-κB in intact cells. PDTC (75 and 150 mg/kg, ip.) inhibits immobilization stress-induced increase in iNOS expression. PDTC administered in combination with NOS inhibitors used in the present study served to indicate the NOS isoform involved in anxiogenic stress stimulus in mice. PDTC enhanced the anti-anxiety effect of AG (iNOS inhibitor) but not that of 7-NI (nNOS inhibitor). PDTC per se has also shown anti-anxiety effect in stressed mice but not in unstressed mice. PDTC pretreatment did not change the antianxiety effect of 7-NI as compared to 7-NI alone. It seems that PDTC pretreatment decreased the effect of 7-NI (40 mg/kg) (because, the numerical value calculated, came out to be less, 125.1); it should be noted that anxiolysis exerted by the combination is greater than vehicle-treated unstressed group, which is of more relevance to us, to be assured that, PDTC pre-treatment has not compromised the antianxiety effect of 7-NI per se.

From all the observations, it is indicated that different inhibitors of nitric oxide synthases behave differently in unstressed and stressed mice tested in same behavioral paradigms of EPM and LDT under same experimental conditions. These observations strengthen the results of experiments, that, pharmacological treatments may behave differently in different forms of anxiety as documented by Belzung and Griebel that even standard treatments like benzodiazepines and serotonin receptor agonists behaved differently in different forms of anxiety. Further, the study fulfills the author’s objectives and answers the study questions in pointwise manner that forms the conclusion of the present study.

Conclusion
The results of the present study (a) suggest the involvement of nNOS in normal short term anxious states and decline its role in stress-induced anxiogenic behavior in mice; (b) the observations encourage the
use of iNOS inhibitors in situations of anxiety, manifested by previous stressful exposures; and (c) it seems that exposure to enduring situations involve underlying activation of iNOS, which may take over from nNOS in the course of sustained development of anxious behavior.

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