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Differential role of nitric oxide (NO) in acute and chronic stress induced neurobehavioral modulation and oxidative injury in rats

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ABSTRACT

The present study evaluated the effects of acute and chronic restraint stress (RS 1 h or 6 h), and their modulation by nitrergic agents on neurobehavioral and oxidative stress markers in rats. Acute RS (1 h or 6 h) reduced open arm entries (OAE) and open arm time (OAT) in the elevated plus maze test — which were attenuated by the NO precursor, L-arginine but not influenced appreciably by the NO synthase inhibitor, L-NAME. These behavioral changes were associated with differential changes in brain NO metabolites (NOx) but consistently reduced GSH and raised MDA levels in comparison to the control group. Following RS 1 h×10 the neurobehavioral suppression and changes in brain oxidative stress markers were less pronounced as compared to the acute RS (1 h) group indicating adaptation. L-arginine pretreatment facilitated this adaptation to chronic RS (1 h). Interestingly RS 6 h×10, induced severe behavioral suppression and aggravation of MDA and NOx levels and L-NAME pretreatment tended to protect against these chronic RS induced aggravations. These results suggest that acute and chronic RS induces duration/intensity dependent neurobehavioral and oxidative injury which are under the differential regulatory control of NO.

1. Introduction

Stressful stimuli are known to disrupt the physiological milieu and complex mechanisms are proposed and the ability of the organism to cope with such aversive stimuli is a crucial determinant of health and disease (Carrasco and VandeKar, 2003; Chrouses and Gold, 1992). Since the introduction of the concept of stress to biomedical research by Hans Selye (Selye, 1936) considerable advances have been made over the last few decades, and cellular and molecular concepts are now being forwarded to explain stress effects. The neurochemical pathways in CNS have been reported to play a crucial role in the regulation of stress responses and the involvement of neurotransmitters and neuromodulators like biogenic amines, aminoacids and peptide and their interactions have been proposed (Ray et al., 1987, 1988, 1991). Further, host factors like age, sex and emotional status are instrumental in deciding the nature and the extent of the impact of such aversive inputs on the biological system (Gulati et al., 2006; Chakraborti et al., 2007, 2008). 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 proposed (Glavin et al., 1991; Henke et al., 1991; Henke and Ray, 1992). Factors like emotionality and prior stress exposure are also known to influence stress susceptibility, and adaptation has been reported after repeated exposures to such aversive stimuli (Kloet et al., 2005; Gulati et al., 2006). Acute and chronic stress exposures are known to elicit differing nature of responses on the biological system, and though the mechanisms for acute stress have been explained, the modalities involved during chronic stress responses are not clearly defined.

Recently, the role of free radicals in the pathogenesis of CNS disorders has been suggested (Calabrese et al., 2007). Reactive oxygen species (ROS) are well recognized for playing a dual role as both beneficial and deleterious molecules. Lower concentrations of these highly reactive species are involved in the regulation of a number of physiological processes whereas overproduction results in oxidative injury which can be an important mediator of damage to cell structures like lipids, membranes, protein and DNA (Valco et al., 2007). Nitric oxide (NO), another 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 (Kojda and Harrison, 1999). The role of NO in several pathophysiological states has been suggested and NO and its modulators are effectively used as therapeutic agents (Kojda and Harrison, 1999; Rand, 1992; Moncada and Higgs, 1993; Zhang and Snyder, 1995; Ricciardolo et al., 2004). Our initial studies have shown that effects of acute restraint stress of short duration may be under the regulatory

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influence of NO (Chakraborti et al., 2007; Gulati et al., 2006, 2007). However, the role of NO in chronic stress mechanisms is not clearly understood and needs to be delineated. 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 different stress paradigms on neurobehavioral and biochemical markers of stress responses in rats. Further, interactions of NO with oxidative stress markers during such aversive conditions were also investigated.

2. Materials and methods

Male Wistar rats (175–200 g), $n=6$ per group, were used for the study. The research protocol was approved by the Institutional Animal Ethical Committee (IAEC) and the experimental procedures are in compliance with national guidelines laid down by the Indian National Science Academy (INSA). The animals had free access to food and water and were housed in standard laboratory conditions. Restraint stress (RS) in plexiglas restrainers (INCO, Ambala) for 1 h or 6 h at room temperature was used as the experimental stressor. Both single (×1) and repeated (×10) schedules were followed in both stress paradigms. All stress (RS) procedures were performed between 9 AM and 3 PM. After stress procedure the animals were observed for behavioral activity in the elevated plus maze test.

2.1. Neurobehavioral studies

Elevated Plus Maze (EPM) test (Pellow et al., 1985): Immediately after the RS procedure termination, the animals were subjected to the EPM test. The animals were placed in the central square of elevated plus maze and observed for 5 min, and the number of open arm entries (OAE) as well as the time spent in the open arms (OAT) were recorded during this 5 min exposure. The results were expressed as percentage by dividing the number of entries in open arms by the total open and closed arm entries. Similarly, the percent time spent in the open arms was also calculated during the 5 min (300 s) exposure in the EPM.

2.2. Biochemical assay

Immediately after termination of RS procedure and subsequent behavioral tests, the rats were decapitated under ether anesthesia. The brain was cleaned with ice cold saline and stored at −80 °C. Brain samples were thawed and homogenized with 10 ml of ice cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates were used to determine the oxidative and nitrosative stress markers viz. MDA, GSH and total nitrates/nitrites (NOx), respectively.

MDA was measured by the method of Okhawa et al. (1979).The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium lauryl sulfate (8.1%) were added to 0.1 ml of processed tissue samples, and heated at 95 °C for 60 min. The mixture was cooled with tap water and 5 ml of n-butanol/pyridine (15: 1 v/v), 1 ml of distilled water was added. The mixture was vortexed and after centrifugation at 4000 rpm for 10 min, the organic layer was separated, and absorbance was measured at 532 nm using a UV–VIS Spectrophotometer (UV 5740 SS, ECIL). Protein estimation was done according to Lowry et al. (1951) and the data were expressed as nmol/mg protein.

Reduced glutathione (GSH) was measured according to the method of Ellman (1959). An equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5-5 dithiobis (2 nitrobenzoic acid) and 0.4 ml of double distilled water was added. The mixture was vortexed and absorbance read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as μmol/g tissue.

For NO assay, NOx (stable NO metabolites) was estimated in brain homogenates. Samples were assayed spectrophotometrically (ECIL, India) using the method of Tracey et al. (1995). Briefly, the brain homogenates were treated with nitrate reductase+NADPH+FAD to convert all nitrates to nitrites. After incubation for 1 h at 37 °C 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% napthyl 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 and data were expressed as nmol/mg of protein.

Table 1
Effect of restraint stress (RS 1 h) and NO modulators on neurobehavioral, brain nitrosative and oxidative stress parameters in rats

Treatment mg/kg (i.p.)	EPM parameters (%)		Brain biochemical parameters		
	OAE	OAT	Nox (nmol/mg pr)	MDA (nmol/mg pr)	GSH (μmol/g tissue)
Control (no RS)	24.9(11.5)	13.5(7.1)	2.3(0.9)	5.3(1.6)	9.7(3.5)
RS1 h×1	8.0(7.2)**	3.0(8.8)*	0.9(0.9)*	9.5(3.1)**	4.1(0.8)**
L-Arg(500)+RS 1 h	18.5(14.0)[b]	8.0(10.8)[b]	2.0(0.6)[a]	3.9(2.5)[a]	7.1(3.3)[a]
L-Arg(1000)+RS 1 h	35.0(26.5)[a]	10.0(18.7)[a]	2.9(1.4)[a]	4.3(1.9)[a]	7.8(2.6)[a]
L-NAME(10)+RS 1 h	11.0(13.8)	3.5(7.8)	0.8(0.6)	10.5(3.1)	3.8(2.0)
L-NAME(50)+RS 1 h	7.0(13.3)	1.5(3.5)	0.6(0.9)	12.3(5.8)	3.3(1.5)[b]
RS 1 h×10	12.1(10.0)	11.0(7.7)	2.0(0.7)	7.1(3.2)	6.3(1.7)*
(L-Arg1000+RS 1 h)×10	17.5(22.4)[d]	10.0(21.7)	2.2(1.2)	6.4(2.4)	8.7(2.0)[d]
(L-NAME 50+RS 1 h)×10	9.5(20.5)	9.5(12.5)	0.7(0.9)	11.0(3.5)	4.6(2.5)

All data are expressed as median (interquartile range); $n=6$, per group;
OAE—Open arm entry; OAT—Open arm time; L-Arg: L-arginine.
*$p<0.01$; **$p<0.001$ (compared to control group).
[a]$p<0.01$; [b]$p<0.05$ (compared to RS1 h×1 group).
[c]$p<0.01$; [d]$p<0.05$ (compared to RS1 h×10 group).

2.2.1. Drugs and chemicals

The drugs and chemicals used were: L-arginine HCl, L-NAME (all from Sigma-Aldrich, USA), FAD, NADPH, nitrate reductase, sulphanilamide, naphthyl ethylene diamine, orthophosphoric acid. L-Arginine and L-NAME were dissolved in normal saline. All drugs were injected intraperitoneally (ip) in a volume of 2 ml/kg following appropriate treatment schedules, viz 60 min for L-arginine and 30 min for L-NAME, prior to RS exposure. The doses of various drugs were selected on the basis of our pilot studies and earlier reports.

2.2.2. Statistics

The behavioral and biochemical data were analysed using the Kruskall–Wallis one way ANOVA for non-parametric data, followed by Mann–Whitney U test (two-tailed) for inter-group comparisons. A p value of at least 0.05 was considered as the level of significance in all statistical tests.

3. Results

Effects of acute and chronic stress of different durations i.e. RS given for 1 h and 6 h were determined on various stress responses in rats. RS for 1 h alone or in combination with various NO modulators significantly altered the number of open arm entries (OAE) as well as the time spent in open arm (OAT) during the 5 min exposure to the EPM test. Overall analysis of data showed that these behavioral changes were significantly different across all groups ($H=19.5$, d.f.=8, $p<0.02$, for OAE and $H=22.3$, $p<0.01$ for OAT, Kruskall–Wallis test). Specifically, the data of the RS exposed group were significantly lower as compared to the control (no RS) group ($p<0.001$ for OAE and

Table 2
Effects of NO modulators on neurobehavioral parameters and brain NO metabolites (NOx) in rats

Treatment mg/kg (i.p.)	EPM parameters (%)		Brain NOx (nmol/mg pr)
	OAE	OAT	
Controls (o RS)	24.9 (11.5)	13.5 (7.1)	2.3 (0.9)
L-Arg (1000)×1	29.0 (14.5)	20.0 (14.8)	3.0 (1.2)
L-NAME (50)×1	20.0 (10.2)	10.0 (6.8)	1.8 (1.1)
L-Arg (1000)×10	26.0 (15.8)	19.0 (9.5)	2.8 (1.1)
L-NAME (50)×10	18.0 (11.8)	13.0 (6.3)	1.9(0.6)

All data are expressed as median (interquartile range); $n=6$ per group.
L-Arg — L-arginine.

$p<0.01$ for OAT, Mann–Whitney *U*-test). 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 ($p<0.05$, normal '*Z*' test for proportions). 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. However, when the rats were repeatedly exposed to RS (1 h/day) for 10 days there was a partial reversal of the behavioral suppression in the EPM test. The mean OAE and OAT in the RS 1 h×10 group were increased by 60% and 96% respectively, as compared to the data of single RS exposed group. The % suppressions of OAE and OAT in this group were not significantly different from that observed in control (no RS) group ($p>0.05$). Pretreatment of rats with L-arginine (1000 mg/kg) further augmented the reversal of behavioral suppression observed after repeated RS 1 h exposure ($p<0.05$ vs. RS 1 h for the OAE data) whereas L-NAME (50 mg/kg) prior to each RS exposure did not affect the response. These data are summarized in Table 1. Administration of these NO modulators, viz. L-arginine and L-NAME, at the doses used, per se, did not produce any appreciable effect on the EPM parameters in non-stressed rats ($p>0.05$, compared to controls; Mann–Whitney "*U*" test; Table 2).

In a separate set of experiments, when rats were exposed to more severe form of stress i.e. RS given for 6 h, a similar neurobehavioral suppression was observed. Overall analysis of the data showed that the OAE and OAT data were significantly different across all groups ($H=34.2$, d.f.=8, $p<0.001$ for OAE; and $H=22.4$; d.f.=8, $p<0.01$ for OAT, Kruskall–Wallis test). There was a reduction in number as well as time spent in the open arms in EPM test. However, the reduction was 33% and 25% in OAE and OAT as compared to that of 67% (for both) after 1 h of RS. Prior treatment with L-arginine (500 and 1000 mg/kg) significantly reversed the EPM parameters ($p<0.01$, for both OAE and OAT data). However, repeated exposure (×10) to RS 6 h completely abolished the number of OAE and hence the OAT in the EPM ($p<0.001$, as compared to the RS 6 h×1 group. Pretreatment with NO modulators, precursor as well as NO synthase inhibitors did not influence these responses in EPM test ($p>0.05$). These data are summarized in Table 3.

Analysis of the brain homogenate data showed that the markers of oxidative stress i.e. MDA, GSH; and NO metabolites (NOx) tested in this study were significantly different across all groups ($H=40.3$, d.f.=8, $p<0.001$ for MDA; $H=40.3$, d.f.=8, $p<0.001$ for GSH; $H=26.7$, d.f.=8, $p<0.001$ for NOx; Kruskall–Wallis test). A single exposure to RS for 1 h resulted in (a) increased MDA levels, and (b) reductions in GSH and NOx levels, in brain homogenates, as compared to the control values ($p<0.001$ for MDA and GSH; $p<0.01$ for NOx, Mann–Whitney *U* test). Prior administration of NO precursor, L-arginine (500 and 1000 mg/kg) significantly prevented these responses to RS, whereas L-NAME did not influence them significantly (Table 1). After repeated exposure, RS 1 h×10, there was a relative reduction in the degree of changes in these biochemical markers i.e. MDA levels were increased by 34% as compared to 79% in RSx1 group as compared to controls, whereas, GSH and NOx levels were reduced by 35% and 13% respectively as compared to 58% and 61% after single RS. Pretreatment with L-arginine (1000 mg/kg) further facilitated, whereas L-NAME (50 mg/kg) was ineffective— rather it tended to attenuate the partial reversal seen after repeated RS. (Table 1).

Interestingly, when the rats were exposed to RS 6 h there was a significant elevation in the NOx levels ($p<0.05$, vs. control) contrary to the reduction observed after RS 1 h, but was accompanied with reduced GSH and elevated MDA levels ($p<0.001$, vs. control) similar to that seen after RS 1 h. Pretreatment with L-arginine (500 mg/kg) significantly attenuated the changes in GSH and the NOx responses to RS 6 h. However, L-NAME (10 and 50 mg/kg) administration prior to RS significantly reduced the NOx levels ($p<0.01$, vs. RS 6 h) without affecting the oxidative stress markers i.e. MDA and GSH ($p>0.05$). Repeated exposure (×10) to RS 6 h markedly elevated the levels of MDA and NOx as compared to controls ($p<0.001$), as well as with those of the RS 6 h (×1) group. This is in contrast to the situation after RS 1 h×10 where reversal of these parameters towards normalcy was observed from that of RS 1 h×1. Interestingly, both L-arginine and L-NAME similarly modulated the responses to repeated RS 6 h, i.e. partially prevented the changes in NOx, GSH, and MDA. These data are summarized in Table 3.

4. Discussion

The results of the present study clearly show that an emotional stressor like RS induces stressor intensity/duration dependent changes in neurobehavioral parameters and brain oxidative stress markers. Acute or chronic RS exposure for 1 h or 6 h induced distinctly different nature of biological responses in our experiments. In repeated as well as single RS studies, the behavioral test and sampling for biochemical assays were done immediately after the termination of the RS procedure thus the released physiological effects of RS were not ignored. The EPM test is a widely used and consistent model to study anxiogenic/anxiolytic responses and our current findings using different durations of RS (1 h or 6 h) reaffirm this concept. Acute RS induced anxiogenic responses manifested as reduced open arm entries and time spent, but surprisingly the neurobehavioral suppression was of lesser magnitude following 6 h RS as compared to that after 1 h. The neurobehavioral suppression was reversed by L-arginine (NO precursor) in a dose dependent manner. The behavioral effects of RS in the EPM test and its modulation by NO-ergic agents are supported by the changes in NOx levels in the brain homogenates of

Table 3
Effect of restraint stress (RS 6 h) and NO modulators on neurobehavioral, brain nitrosative and oxidative stress parameters in rats

Treatment mg/kg (i.p.)	EPM parameters (%)		Brain biochemical parameters		
	OAE	OAT	Nox (nmol/mg pr)	MDA (nmol/mg pr)	GSH (μmol/g tissue)
Control (no RS)	24.7(10.3)	12.8(5.3)	2.7(0.9)	4.1(1.9)	8.8(2.3)
RS 6 h×1	16.5(13.0)*	10.8(9.1)	3.4(2.5)*	10.9(5.5)**	3.8(1.6)**
L-Arg(500)+ RS 6 h×1	35.5(9.0)[a]	13.0(13.9)	2.7(1.1)	13.0(5.0)	4.8(1.8)[b]
L-Arg(1000)+ RS 6 h×1	39.6(11.3)[a]	16.6(11.3)	3.5(1.7)	13.0(4.0)	6.0(2.8)[a]
L-NAME(10)+ RS 6 h×1	19.2(16.3)	17.0(11.0)	1.7(0.8)[a]	10.3(2.0)	3.9(2.0)
L-NAME(50)+ RS 6 h×1	16.3(23.9)	24.7(15.0)	0.9(0.9)[a]	10.3(4.5)	3.0(1.6)
RS 6 h×10	0**	0**	7.3(4.9)**	17.3(3.7)**	3.5(1.6)**
(L-Arg1000+ RS 6 h)×10	0	0	5.1(1.6)[c]	11.2(6.4)[c]	4.8(1.5)
(L-NAME 50+ RS 6h×10	0	0	4.0(2.1)[c]	11.8(2.6)[c]	6.4(2.0)[c]

All data are expressed as median (interquartile range); $n=6$, per group;
OAE—Open arm entry; OAT—Open arm time; L-Arg: L-arginine.
*$p<0.01$; **$p<0.001$ (compared to control group).
[a]$p<0.01$; [b]$p<0.05$ (compared to RS 6 h×1 group).
[c]$p<0.05$ (compared to RS 6 h×10 group).

these rats. The CNS is highly susceptible to oxidative damage and the association of brain lipid peroxidation and restraint stress has been reported in our earlier study (Pal et al., 2006). The similar elevations in brain MDA and reductions in GSH levels, in our present experiments, emphasize that emotional stressors like RS result in imbalance between pro-oxidant and anti-oxidant factors in the brain. The parallel reversals in brain NOx (marker of nitrosative stress), MDA and GSH levels (markers of oxidative stress), after L-arginine, are strongly indicative of RNS–ROS interactions during RS 1 h, and also suggest a probable anti-oxidant role for NO during such situations. Similar antioxidant effect of NO was also described by Hummel et al. (2006). Interestingly, as observed with 1 h RS, single exposure to 6 h RS resulted in neurobehavioral suppression and oxidative stress but, in contrast, was accompanied with elevated levels of NOx. This could be due to induction of iNOS following longer duration stress (6 h), as Madrigal et al. (2001) have also shown enhanced expression of iNOS in brain cortex after 6 h of acute stress.

Repeated exposure to RS 1 h led to attenuations of neurobehavioral suppression and biochemical responses which could have been due to the tolerance to stressful stimuli. Adaptation to stress is a physiological autoregulatory mechanism which controls the impact of the stressor on the biological system and chronic stress exposures are known to induce adaptive responses. Prior administration of L-arginine facilitated the adaptation to chronic RS (1 h). There are reports of similar attenuation of neurohormonal stress response associated with habituation of the HPA-axis on repeated exposure to stress (Fulford and Harbuz, 2005). Our results are also in agreement with Fontella et al. (2005) who showed a consistent increase in MDA and reduced total antioxidant reactivity (TAR) in the hippocampus after repeated RS for 1 h/day for 40 days as compared to the controls. In the present study, whereas repeated RS for 1 h (×10) showed reversal of neurobehavioral responses in the EPM, this was not evident in the RS 6 h×10 model of chronic stress. Surprisingly, repeated RS 6 h×10 days resulted in intense neurobehavioral suppression accompanied with aggravation in the levels of NOx which could be due to the prolonged stimulation of iNOS and hence accumulation of NO metabolites. There is substantial evidence that in certain conditions the bioavailability of NO is reduced and loss of physiological function occurs because of oxidative inactivation by excessive production of superoxide anions (Kojda and Harrison, 1999; Olivenza et al., 2000). Both superoxide and NO are highly reactive entities which produce peroxynitrite and cause cellular damage (Marechal et al., 2007). This could be a plausible explanation for the concurrent parallel increase in MDA (a marker of lipid peroxidation) and reduction in GSH (a reliable measure of antioxidant defense observed) after repeated stress for 6 h and failure of reversal of stress responses after administration of L-arginine, seen in our experiments. Moreover, there are reports that there is a reversal of function of NO from antioxidant at lower concentration, to pro-oxidant at higher concentration (Gulati et al., 2007). Our present experimental data indicate that such failure of regulatory adaptive mechanisms may have occurred during such severe form of stress (RS for 6 h×10), wherein the plasticity of stress mechanisms were completely disrupted. However, this is a novel finding that nitrergic modulation of stress responses depends on the duration and intensity of the stressor. There was a parallel increase in the oxidative and nitrosative stress markers after 6 h RS whereas opposite directional changes i.e. increase in MDA and reduced NOx, were seen in these parameters after 1 h RS. Further, there was a paradoxical aggravation of neurobehavioral responses and oxidative stress markers after repeated exposure to 6 h rather than adaptation as observed after the RS 1 h×10 group. Contrary to the expectations, both L-NAME (NOS inhibitor) as well as L-arginine (NO precursor) similarly modulated the biochemical responses to RS (6 h)×10. The interesting observation of reductions in NOx levels by L-arginine may be due to the diversion of biosynthetic/metabolic pathway to form agmatine which is reported to downregulate iNOS (Halaris and Plietz, 2007). Further, Moris (2007) also suggested that transformation of L-arginine to NO or agmatine depends on crucial factors like cell type, age, diet, state of health and disease etc. Our results also reaffirm the contention that L-arginine may have exhibited differential responses depending on the varying stressor (RS) durations and intensities i.e. 1 h or 6 h and single or repeated exposures.

Taken together, the present results suggest that acute and chronic stress responses are differentially regulated by nitric oxide. Further, both RNS and ROS may play a crucial role in the regulation of and adaptation to stressful exposures, and, whereas, physiological levels of NO (probably due to cNOS activity) as well as its anti-oxidant potential protect against stress, breakdown in adaptive mechanisms could be due to excessive NO formation (probably via iNOS) and its combination with deleterious oxidative forces.

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