



Experimental studies on possible regulatory role of nitric oxide on the differential effects of chronic predictable and unpredictable stress on adaptive immune responses



Tarun Thakur, Kavita Gulati*, Nishant Rai, Arunabha Ray

Department of Pharmacology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110007, India

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ABSTRACT

The present study was designed to investigate the effects of chronic predictable stress (CPS) and chronic unpredictable stress (CUS) on immunological responses in KLH-sensitized rats and involvement of NOergic signaling pathways mediating such responses. Male Wistar rats (200–250 g) were exposed to either CPS or CUS for 14 days and IgG antibody levels and delayed type hypersensitivity (DTH) response was determined to assess changes in adaptive immunity. To evaluate the role of nitric oxide during such immunomodulation, biochemical estimation of stable metabolite of nitric oxide (NOx) and 3-nitrotyrosine (3-NT, a marker of peroxynitrite formation) were done in both blood and brain. Chronic stress exposure resulted in suppression of IgG and DTH response and elevated NOx and 3-NT levels, with a difference in magnitude of response in CPS vs CUS. Pretreatment with aminoguanidine (iNOS inhibitor) caused further reduction of adaptive immune responses and attenuated the increased NOx and 3-NT levels in CPS or CUS exposed rats. On the other hand 7-NI (nNOS inhibitor) did not significantly affect these estimated parameters. The results suggest involvement of iNOS and lesser/no role of nNOS during modulation of adaptive immunity to stress. Thus, the result showed that predictability of stressors results in differential degree of modulation of immune responses and complex NO-mediated signaling mechanisms may be involved during responses.

1. Introduction

Stress is a complex phenomenon that triggers multiple physiological responses in the nervous, endocrine, and immune systems referred to as 'stress response'. Stressors are known to influence the physiological milieu and disturb the homeostasis resulting in either disease states or development of adaptive mechanisms [1]. These coordinated responses are composed of altered behavior, immunity, autonomic function and secretion of multiple hormones. Effects of stress on an organism depend upon various factors viz. type, intensity, and the duration of a particular stressor or physiological factors like strain, gender and age of the subjects [1–5]. In addition, differential responses to acute and chronic stress have been documented on neuroendocrine, visceral and immune systems. The immune system is particularly susceptible to stressors and could result in inflammatory, immunological, infectious and neoplastic disorders. It is widely accepted that acute stress tends to enhance immune functioning, whereas chronic stress results in suppressed immune response [6]. Further, predictability of stressor can also differentially affect various immune responses to stress and the mechanisms involved therein are not well defined.

Nitric oxide (NO), which was initially discovered in the vascular endothelium, is now recognized as a unique messenger molecule in CNS and immune systems and several physiological functions have been attributed to NO. It has also been implicated in various pathological states and its role in stress has been proposed. NO is generated from precursor, L-arginine which is acted upon by NO synthase enzymes and at least three functional forms of NO synthase enzymes are known - endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) [7]. Biological signaling by NO can be categorized into direct and indirect actions (cGMP-mediated and non cGMP-mediated respectively). The direct actions of NO are considered to be cGMP mediated and involve the constitutive NO synthases (nNOS and eNOS), which leads to execution of terminal responses like smooth muscle relaxation, neuronal transmission and inhibition of platelet aggregation, to name a few. But signaling between immune and inflammatory cells by NO is considered largely to be indirect, where NO mediated formation of RNOS may propagate the actions of NO [8]. So, indirect actions of NO are considered to be relative more important, while associating its effect(s) on the immune system. However, there is a paucity of studies which can indicate/establish the relative involvement of specific eNOS, nNOS and

* Corresponding author at: Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110 007, India.
E-mail address: kavgul2002@yahoo.com (K. Gulati).

iNOS during immunomodulation in response to chronic stress.

As bidirectional circuit exists in case of CNS-immune interactions the role of nNOS can influence the outcomes of immune responses and is not well defined. So in the present study pharmacological inhibition of nNOS and iNOS was done to gain insight into possible mechanisms involving NO (specific) during immune responses to chronic stress. Gilhotra and Dhingra [9] employed sildenafil and suggested involvement of NO-cGMP pathway in antianxiety effect of aminoguanidine in stressed mice. However, the NO-signal transduction mechanisms involved during immunomodulation in response to chronic stress are far from clear and needs to be delineated. The present study evaluated the modulatory role of NO during chronic stress-induced immunological responses in KLH-sensitized rats and discussed complex signaling pathways mediating such adaptive immune responses.

2. Materials and methods

2.1. Animals

Inbred male wistar rats (180–250 g) were used in the study and each experimental group comprised of 6 animals. They were housed under standard laboratory conditions ($22 \pm 2^\circ\text{C}$). All procedures related to maintenance and experimentation were in accordance with guidelines *Care and Use of Animals in Scientific Research* prepared by INSA, New Delhi (INDIA) and the study protocol had the approval of Institutional Animal Ethics Committee (IAEC).

2.2. Drugs and chemicals

NO synthase inhibitors.

- (a) iNOS inhibitor - aminoguanidine (50 and 100 mg/kg, i.p.).
- (b) nNOS inhibitor - 7-nitroindazole (30 and 60 mg/kg, i.p.).

The drugs mentioned above were procured from Sigma-Aldrich, USA. The ELISA kits used in the present study were from Shibayagi, Japan for KLH-specific IgG and Cusabio, China for 3-NT. All the other chemicals used in this study were of analytical grade procured from reputed standard manufacturer (Sisco research laboratories, India).

2.3. Instruments

Some of the major instruments used during the current study were: iMark™ microplate absorbance reader, BIO-RAD, USA; Immunowash-1575, BIO-RAD, USA; Spectramax M3, UGO basile biological research apparatus Plethysmometer-7140, Italy; Molecular Devices, USA.

2.4. Stress protocols

Rats were randomly assigned to two experimental protocols of stress exposure. One set of animals were subjected to chronic predictable stress (CPS) and other to chronic unpredictable stress (CUS) for 14 consecutive days along with vehicle administration. The choice of CPS and CUS protocols was guided by growing appreciation of the fact that repeated application of the same or different stressor can have differential effects on hippocampal and amygdaloid neurons [10,11]. Therefore, stress protocol comprising of a same or combination of different stressors was used to study immune responses and the mechanisms involved therein.

2.4.1. Chronic predictable stress (CPS)

For CPS, same kind and intensity of stress was given daily-fixed implementing time schedule was followed. Rats were immobilized in specific Plexiglas restrainers (INCO, Ambala) without inflicting any pain for 1 h at room temperature. This restraint or immobilization technique is widely validated animal model of stress because it induces

Table 1

Sequence of stressor-types that constituted the CUS procedure.

Day	Stressor type-utilized	Duration
1.	Immobilization restraint stress	1 h
2.	Foot shock	3 min
3.	Cold stress	1 h
4.	Food and water deprivation overnight	18 h
5.	Social isolation overnight	18 h
6.	Foot shock	3 min
7.	Food and water deprivation overnight	18 h
8.	Social isolation overnight	18 h
9.	Immobilization restraint stress	1 h
10.	Cold stress	1 h
11.	Social isolation overnight	18 h
12.	Food and water deprivation overnight	18 h
13.	Foot shock	3 min
14.	Immobilization restraint stress	1 h

activation of sympatho-adrenal-medullary system, the HPA axis, elevation of blood pressure and heart rate which accompanies a stressful situation [12]. All stress procedures were performed between 9 AM and 1 PM.

2.4.2. Chronic unpredictable stress (CUS)

For CUS paradigm, rats were subjected to one stressor daily from below mentioned five different stressors for 14 days as per Table 1.

- i) Immobilization stress for 1 h.
- ii) Foot shock (in foot shock chamber 150 V, 2 mA, for 3 min).
- iii) Cold stress (exposure in cold chamber at $4\text{--}6^\circ\text{C}$ for 1 h).
- iv) Food and water deprivation overnight.
- v) Social isolation overnight.

The chronic unpredictable stress protocol was a modified mix from models used previously [13–16].

2.5. Immunological studies

Rats were immunized subcutaneously with 1 mg of keyhole limpet hemocyanin (KLH) in 0.4 ml antigen preparation (FCA and PBS in equal ratio 1:1) near the tail region on day 0. KLH is a highly immunogenic and stable T-cell dependent antigen. After various drug treatments from day 0 to day 14, the rats were challenged on day 14 with 1/10th of KLH-immunizing dose i.e. 100 µg subcutaneously. After 24 h paw volumes were measured and the animals were sacrificed, terminal blood collection was done by heart puncture and brains were removed for various biochemical and immunological assays as per protocol.

2.5.1. Antibody titer assay

Antibodies are the effectors of humoral immunity which are generated by B-cells and eliminate antigen by complement fixation, antibody directed cell cytotoxicity etc. IgG is the key player in the humoral immune response and is a major immunoglobulin in blood and extracellular fluid and can be retained in the body for long [17]. This assay was carried out to assess the humoral immune response in stress exposed rats. Serum anti-KLH IgG concentration was determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit as per the manufacturer's instruction manual. All the kit contents were brought to room temperature. KLH-coated plate was washed three times with wash buffer. 50 µl of standard or diluted samples were placed in preselected wells for each. After placing the standards and the samples the plate was shaken gently on a plate shaker, sealed and incubated for 1 h at $20\text{--}25^\circ\text{C}$. Wash was given three times with wash buffer on an automated microplate washer (BIO-RAD Immunowash-model 1575). 50 µl of HRP-conjugated anti-rat IgG antibody was added to each well. Plate was sealed and incubated for 1 h at $20\text{--}25^\circ\text{C}$ and

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