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Original article

Psychosocial stress inhibits additional stress-induced hyperexpression of NO synthases and IL-1β in brain structures

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ABSTRACT

Background: The aim of this study was to compare the expression of interleukin-1β (IL-1β), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) in the prefrontal cortex (PFC), hippocampus (HIP) and hypothalamus (HT) during chronic crowding (CS) (psychosocial) and restraint (RS) (physico-psychological) stress. Adaptational changes of these stress mediators to a subsequent acute RS, in two models of chronic stress were investigated.

Methods: Rats were crowded (24 in one cage) or restrained in metal tubes for 10 min twice a day for 3, 7, and 14 consecutive days and decapitated. For determination of adaptational changes the chronically crowded and restrained rats 24 h after the last stress session were subjected to a single 10 min RS. The IL-1β, nNOS and iNOS protein levels in brain structures samples were analyzed by Western blot procedure.

Results: Chronic CS for 3 days did not markedly change the subsequent acute stress induced expression of nNOS, iNOS and IL-1β protein level in PFC and iNOS protein level in HT. CS markedly decreased the expression of nNOS, iNOS and IL-1β in HIP. By contrast, parallel chronic RS, significantly increased the subsequent acute stress-induced expression of iNOS and IL-1β in PFC and considerably increased iNOS level in HT.

Conclusion: Chronic psychosocial stress, may protect against possible harmful action of hyperproduction of iNOS and iNOS derived nitric oxide (NO) mainly in PFC and HIP. By contrast, chronic physico-psychosocial stress may strongly potentiate additional stress-induced harmful effects of NOS and IL-1β hyperproduction.

Introduction

The brain sensitive structures the prefrontal cortex (PFC), hippocampus (HIP) and hypothalamus (HT) as part of the limbic system play important roles in the regulation of the major stress hormonal axis in the body [1,2]. Profound biochemical changes are involved as pathogenic factors in several neuropsychiatric diseases such as depression [3–5]. Reactive stressors, that increase the demand on central brain system through a sensory stimulus, (such as pain, injury or an immune challenge) are preferentially transmitted to brainstem and specific hypothalamic nuclei which have direct connection to the hypothalamus paraventricular nucleus (PVN) [6]. In experimental studies in a wide variety of animal models the involvement of social and physical environmental stress to the development of biological substrates of diseases were investigated. However, many animal models of stress do not sufficiently reflect both the etiology and the symptomatology of human stress-related diseases. Most of the stressors used in the animal models have little or no relationship with the biology of the species that an animal may meet in its everyday life situations [7,8].

In laboratory animals, a variety of the experimental methods, most frequently immobilization and restraint are used in stress research. Immobilization is categorized as physical/psychological stressor. Restraint may be considered as example of a more psychological than physical stressor in which the stress response may result rather from the situation (closing in a tube) than from direct noxious stimuli. Stress reaction not only prepares an individual for the consequences of dangerous situations and return to homeostasis but may induce long-time adaptive responses. Animals previously exposed to chronic stressor when presented with acute, the same (homotypic) or novel (heterotypic) stressor exhibit diminished, normal or increased adapted stress response compared to animals exposed solely to that acute stressor [9–11]. The blunted response to homotypic stressor after repeated exposure is referred as "stress adaptation" and is considered as a key protective mechanism against harmful effects of prolonged stress exposure [9,12,13].

Negative social relationships seems to be a more potent source of chronic stress and disease compared with traditional psychological

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and physical stress models. Psychosocial relations are powerful modulators of the stress response because they are conditioning factors for health in humans and animal kingdom [7,14]. Chronic stress can modulate central cytokines under experimental conditions in absence of inflammation [10,15,16]. Peripheral and central induction of interleukin-1β (IL-1β) can markedly affect the neurohormonal effects of chronic stress. Physical and psychological stress elevate plasma level of several cytokines, including Il-1β, in animals and humans. Cytokines in the periphery can activate the afferent vagus nerve, which transmit neuronal signals *via* nucleus tractus solitarius (NTS) to higher brain centers, the HT and amygdala. Blood-borne cytokines can also reach the brain directly *via* leakage blood-brain barrier (BBB) or by induced synthesis and release of second messengers prostaglandins (PG), and NO which can influence neurohormonal activity [17].

Cytokine levels in the periphery reflect their synthesis in brain structures [18]. Some psychological and physical stressors can increase IL-1β level which may have neuroendocrine and long-term implications for health [19–21]. IL-1β elevated in stress conditions can markedly affect neurotransmission within emotional regulatory brain circuits and can dysregulate the hypothalamic-pituitary adrenal (HPA) axis, manifested in depressed patients [3,22–25]. Blockade of IL-1β is sufficient to block depressive behavioral and cellular responses resulting from exposure to chronic stress [26]. IL-1β is constitutively expressed in macrophages and microglia, the resident macrophages of the CNS which are ubiquitous throughout the brain, with the highest concentration in the HIP [27]. IL-1β is a key factor in the pro-inflammatory response to psychological stress [28,29] and psychological stressors increase pro-inflammatory IL-1β level in blood and brain tissue [10,30].

Nitric oxide (NO) is a pleiotropic molecule that regulates physiological functions especially in the brain. Overexpression of neuronal NO synthase (nNOS) in the brain is essential for chronic stress-induced modulation signaling in wide range of physiological and pathological conditions [20,31–33].

Brain nNOS exists in differential subcellular localization in particulate and soluble forms and participates in modulation of learning, memory and neurogenesis and pathological condition. nNOS constitutes the predominant source of NO in neurons in synaptic spines. Cytokines are able to evoked inducible NO synthase (iNOS) expression which can be increased by exposure to stress [34]. However, iNOS has been found constitutively expressed in several brain region. In the CNS, astrocytes, microglial cell and macrophages synthesize NO mainly following iNOS expression by different stimuli [35]. Also neurons and endothelial cell can express iNOS and release NO which modulates synaptic release from presynaptic terminals. The stress-induced high output of iNOS is implicated in many cell systems in the stress-related brain structures. The central mechanism of involvement of NO synthase and IL-1β in the adaptation to prolonged stress remain not elucidated.

The aim of this study was to compare the role expression of IL-1β, nNOS and iNOS in PFC, HIP and HT during chronic crowding (CS) psychosocial and restraint physico/psychological stress (RS), which may activate both similar and or different neurobiological pathways in brain structures. The further aim of this study was to examine the adaptational changes of these stress mediators to a subsequent acute RS in two models of chronic stress.

Materials and methods

Animals

Unanesthetized male Wistar rats (6 weeks old, weighing 190–220 g obtained from Charles River Laboratories, Sulzfeld, Germany) were used in the experiments. The animals were housed in cages (52 × 32 × 20 cm) with unlimited access to commercial food and tap water. The animal room was maintained on standard laboratory conditions: an artificial 12-h light/dark cycle (lights on from 7 a.m. to 7 p.m.) and at constant temperature 22 ± 2 °C. Before the onset of the experiments, the animals were given 1 week of habituation period. All the procedures were approved by Local Bioethics Commission for Animal Experiments at the Institute of Pharmacology, Polish Academy of Sciences in Kraków and met the requirements of the European Council Guide for the Care and Use of Laboratory Animals (86/609/EEC).

Experimental procedures

The animals were randomly assigned to control groups or groups subjected to either crowding or restraint stress. To avoid circadian variability, acute stress protocols were performed at light cycle between 10 and 12 a.m.

Crowding stress (CS)

The animals were divided into three groups. The first group consisted of the animals which were kept 5 per cage and were not subjected to any stress (control group). The rats from the second group were kept 24 in one cage (crowding stress) for 3, 7 or 14 consecutive days. The animals from the third group were subjected to crowding stress for 3, 7 or 14 days and after stress period termination they were restrained in metal tubes, with ample holes for ventilation, for 10 min (single acute restraint stress) and then decapitated immediately (0 h), 1, 2 or 3 h later.

Restraint stress (RS)

The animals were divided into three groups. Animals from the first group were housed 5 per cage and were not stressed (control group). The animals from the second group were restrained in the metal tubes for 10 min twice a day for 3, 7 or 14 consecutive days. The break between stress sessions was at least 8 h. The animals from the first and from the second group were decapitated on the day of the experiment. The third group consisted of the animals repeatedly restrained 2 × 10 min for 3, 7 or 14 consecutive days which, 24 h after the last restraint session, were subjected to single 10 min acute restraint and decapitated immediately (0 h), 1, 2 and 3 h later. After decapitation, the brains were removed from the skulls and three whole structures: PFC, HIP and HT were excised on an ice-cold glass plate, immediately frozen on dry ice and stored at −70 °C until assayed.

SDS-PAGE gel electrophoresis and western blot

Protein extracts were prepared according to Gądek-Michalska [36]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot procedures were performed as it was described in our previous study [11]. In the present study IL-1β, nNOS and iNOS protein levels in brain structures samples were determined. The following antibodies were used: primary rabbit anti-nNOS (neuronal nitric oxide synthase) (1:400) and anti-iNOS (inducible nitric oxide synthase) (1:400) polyclonal antibodies and mouse anti-β-actin monoclonal antibody (1:10,000), then goat anti-rabbit (1:10,000) or goat anti-mouse (1:2000) horseradish peroxidase-conjugated secondary antibody, provided by Santa Cruz Biotechnology. For IL-1β determination the following antibodies were used: anti-IL-1β polyclonal primary antibody (1:5000, AbD Serotec) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:20,000, Santa Cruz Biotechnology). The optical density of appropriate bands was quantified by densitometric analysis of blots using Image Gauge